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Award Number: DAMD17-00-1-0115

TITLE: Targeting of Adenovirus Vectors to Breast Cancer Mediated by Soluble Receptor-Ligand Fusion Proteins

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REPORT DATE: June 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Annual (15 May 2001 - 14 May 2002)

4. TITLE AND SUBTITLE
Targeting of Adenovirus Vectors to Breast Cancer Mediated by Soluble Receptor-Ligand Fusion Proteins

5. FUNDING NUNUMBER
DAMD17-00-1-0115

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
The use of adenovirus (Ad) vectors for cancer gene therapy is currently limited by several factors, including broad Ad tropism associated with expression of coxsackievirus and adenovirus receptor (CAR) in normal human tissues, and limited CAR levels in tumor cells. To target breast cancer cell types, we proposed using soluble CAR (sCAR) ectodomain fused with a ligand to simultaneously achieve both blocking of CAR-dependent tropism and Ad infection through a novel receptor overexpressed in target cells. In this regard, the use of α_v-integrins, aminopeptidase N (APN), and c-*erb*B-2 oncoprotein to serve as alternative to CAR might improve Ad infection efficiency and specificity for breast cancer cells. To test this hypothesis we engineered sCAR-ligand proteins containing RGD-4C, NGR peptide, or C6.5 anti-c-*erb*B-2 scFv as targeting moieties. We demonstrated that sCARfRGD and sCARfC6.5 mediated specific Ad binding and efficient CAR-independent infection via integrins and c-*erb*B-2, respectively. In contrast, sCARfNGR protein did not show any improvements in Ad infection of APN-positive cells. Both sCARfRGD and sCARfC6.5 targeting adapters provided up to 120-fold Ad gene transfer enhancement to breast cancer cells compared to sCARf control protein. The use of targeting adapters may augment Ad vector potency for therapeutic gene delivery to breast cancer.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer, cancer therapy, gene therapy, adenovirus, targeting, scar-ligand proteins	33
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION

The use of adenovirus (Ad) vectors for cancer gene therapy applications is currently limited by several factors, including broad Ad tropism associated with the widespread expression of *c*oxsackievirus and *a*denovirus *r*eceptor (CAR) in normal human tissues, as well as limited levels of CAR in tumor cells. To solve these limitations of Ad vectors we propose to target viral infection to the receptors associated with malignant cell transformation. To target Ad to breast cancer cell types, we have proposed using soluble CAR (sCAR) ectodomain fused with a ligand to block CAR-dependent native tropism and to simultaneously achieve infection through a novel receptor overexpressed in target cells (1). We have previously demonstrated that incorporation of an RGD-4C peptide in the HI loop of the Ad fiber knob results in the efficient transduction of primary tumor cells due to the expanded ability of the virus to utilize α_v-integrins during the cell entry process (2). The NGR peptide motif has been shown to target the specific isoform of an aminopeptidase N (CD13), which is expressed in angiogenic tumor blood vessels but not in normal kidney and myeloid cells (3). Amplification of HER-2/*neu*, known as c-*erb*B-2, was found in 30% of breast cancers with as many as 10^6 receptors/cell (4) while normal cells have as few as 10^4 receptors per cell. Thus, the use of α_v-integrins, aminopeptidase N (APN), and c-*erb*B-2 oncoprotein as alternative Ad primary receptors might augment efficiency and specificity of viral infection for breast cancer cell types. To test this hypothesis we engineered sCAR-ligand adapter proteins containing an RGD-4C peptide, NGR peptide, or C6.5 anti-c-*erb*B-2 scFv as targeting moieties. In this report, we describe the study of these novel adapter proteins for their ability to bind the relevant cellular receptors and to target Ad infection to breast cancer cell types.

BODY

Task 2. Evaluate the ability and efficacy of sCAR-ligand fusion proteins for targeted gene delivery to breast carcinoma cells *in vitro.*

Evaluation of ability of sCAR-ligand fusion proteins to bind to the receptors overexpressed on breast cancer cells using binding assays.

The expression levels of CAR and α_v-integrins, a putative receptor for the RGD-4C motif, were determined for a number of established breast adenocarcinomas and described in previous report. Here we demonstrated expression of APN, which was shown to serve as receptor for the NGR motif (5) for a set of cell lines established from breast cancer and endothelial HUVEC cells. The screening of ten breast cancer cell lines revealed a significant level of APN only on MDA-MB-468 cells. Fig. 1 shows representative results of indirect immunofluorescence assay for APN expression in MDA-MB-468, MCF-7, MDA-MB-435S, and HUVEC cells.

The use of sCARfRGD and sCARfNGR adapter proteins may augment infection efficiency by facilitating virus binding to cellular integrins and APN, respectively, in breast cancer cells normally refractory to Ad due to lack of CAR To test this hypothesis we studied the ability of sCARfRGD and sCARfNGR to mediate binding of ^{3}H-radiolabeled Ad to MB-468, MCF-7, MB-435S breast cancer cells and endothelial HUVEC cells. To determine the improvements in virus cell binding ^{3}H-Ad was complexed with dilutions of sCARfRGD, sCARfNGR, or sCARf protein as a control and allowed to bind cells at 4°C to prevent virus internalization. Detected cell-bound radioactivities were dependent on sCAR-ligand protein dose and were significantly higher for ^{3}H-Ad complexed with sCARfRGD compered to ^{3}H-Ad alone. As shown in Fig. 2, Ad complexed with sCARfRGD resulted in 2-, 9-, 60-, and 2-fold increase of ^{3}H-Ad binding to MB-468, MCF-7, MB-435S, and HUVEC, respectively. In contrast, virus complexed with either sCARfNGR or sCARf control protein inhibited virus cell binding compared to Ad alone. These data clearly demonstrate that, in contrast to sCARfNGR, the sCARfRGD adapter protein is capable of enhancing virus binding to cellular integrins and confirm that formation of the Ad/sCARfRGD complexes provide improved of Ad binding to breast cancer cells.

To further refine and extend this approach for particular breast cancer cell types we have engineered the sCARfC6.5 adapter containing the C6.5 scFv with binding specificity for c-*erb*B-2 oncoprotein (6). To validate sCARfC6.5 protein binding to cellular c-*erb*B-2 we employed an indirect immunofluorescence assay. The sCARfC6.5 protein was allowed to bind AU-565 breast cancer cells overexpressing c-*erb*B-2 and then was detected by incubation with a primary anti-CAR RmcB antibody followed by a secondary antimouse fluorochrome-conjugated antibody. The MDA-MB-468 breast

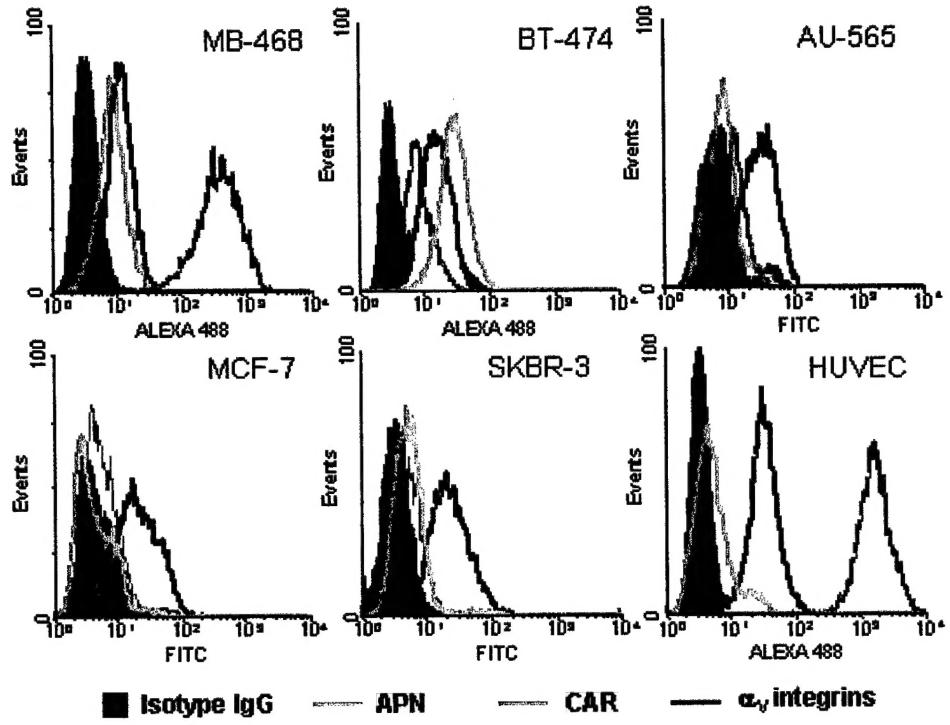

Fig. 1. Expression of APN, CAR, and α_V-integrins in breast cancer cell lines. The cell lines MDA-MB-468, BT-474, AU-565, MCF-7, SK-BR-3, and endothelial cell line HUVEC were analyzed for expression of APN, CAR, and α_V-integrins by indirect immunofluorescence assay using anti-CD13 WM15, anti-CAR RmcB, and anti-α_V-integrins MAB1953 monoclonal antibodies, respectively. Positive staining for APN (*blue line*), CAR (*gray line*), and α_V-integrins (*black line*) is seen relative to an isotype control IgG (*spike filled in black*). Representative data from two independent experiments are shown.

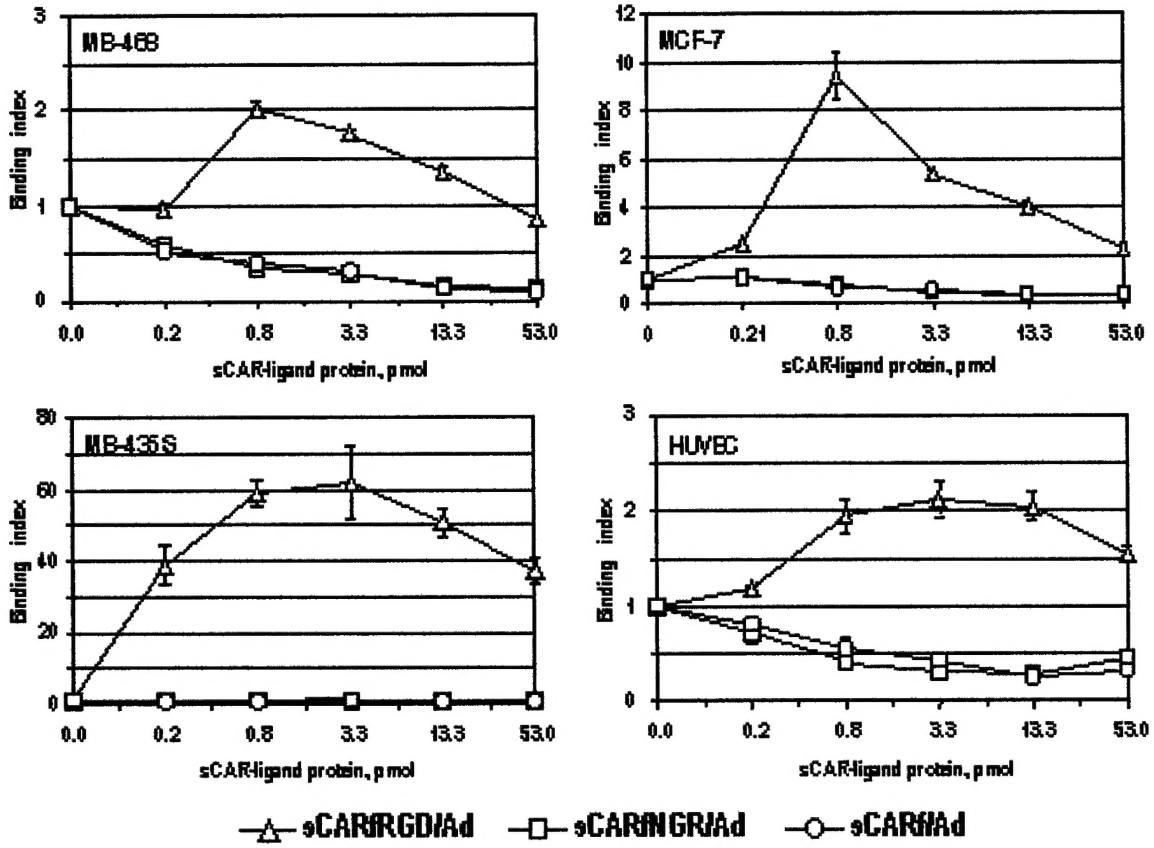

Fig. 2. Comparison of ^{3}H-labeled Ad binding to MDA-MB-468, MCF-7, MDA-MB-435S, and HUVEC cells. ^{3}H-labeled Ad was preincubated for 30 min at room temperature with different amounts of sCARfRGD, sCARfNGR or sCARf as control. ^{3}H-Ad/sCAR-ligand mixtures (10^{5} cpm per sample) were then added to cells aliquots (10^{6}) and allowed to bind for 1 h at 4°C. Bound radioactivity was determined after pelleting the cells by centrifugation. Binding indices were calculated from the ratio of the mean bound radioactivity of ^{3}H-Ad preincubated in presence of sCAR-ligand versus ^{3}H-Ad preincubated in absence of sCAR-ligand protein. Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

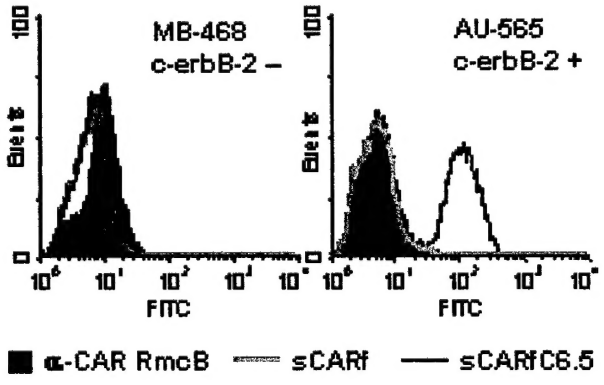

Fig. 3. Confirmation of sCARfC6.5 protein binding to cellular c-*erb*B-2. Trimeric sCARfC6.5 and sCARf fusion proteins were incubated with either c-*erb*B-2-positive AU-565 or c-*erb*B-2-nagative MB-468 cells. The sCAR fusion proteins bound to cells were probed with anti-CAR RmcB MAb and then detected with secondary Alexa 488-labeled goat antimouse antibodies. Binding of sCARfC6.5 protein *(black line)* to c-*erb*B-2-positive AU-565 cells is seen because of the positive staining relative to sCARf control protein *(gray line)* or anti-CAR MAb alone *(spike filled in black)*. Representative data from two independent experiments are shown.

cancer cells, shown to be c-*erb*B-2-negative, were used as a control. As shown in Fig. 3, incubation of AU-565 cells, naturally low in CAR (Fig. 1), with sCARfC6.5 protein increased cell binding of anti-CAR MAb. In contrast, neither the incubation of AU-565 cells with sCARf control protein nor the incubation of sCARfC6.5 with MDA-MB-468 c-*erb*B-2-negative cells revealed any increase of anti-CAR antibody binding compared with control. Thus, we demonstrated the C6.5 scFv that was incorporated in the context of the fusion protein retained its functional ability to recognize cellular c-*erb*B-2 oncoprotein, which enabled sCARfC6.5 protein binding to c-erbB-2-positive cells.

Confirmation of specificity of ligand-receptor binding by blocking with free ligand or specific anti-EGFR monoclonal antibodies in competition-binding assay.

We previously showed that Ad complexed with an sCAR-EGF adapter protein overcomes the barrier of inefficient gene transfer to specific cancer cell types (1). As illustrated in Fig. 4, augmentation of radiolabeled Ad binding to EGFR-positive A-431 cells mediated by sCAR-EGF adapter could be competitively blocked by preincubation of the cells with either human EGF or anti-EGFR MAb, confirming specificity of sCAR-EGF binding to EGFR. In this study we have demonstrated that highest level of Ad binding to MB-468 and MCF-7 cells was achieved at 0.8 pmol of sCARfRGD protein per $1.5x10^8$ vp while 3.3 pmol was required to reach maximum for MB-435S and HUVEC cells (Fig. 2). It is noteworthy that increases in the sCARfRGD/virus ratio proved inhibitory to binding, likely due to excess of free sCARfRGD protein and its competition for integrin binding.

Demonstration of capacity of sCAR-ligand fusions to target Ad to receptors on breast cancer cells using gene transfer assay. Determination of optimal sCAR-ligand/Ad molar ratio that gives maximal gene transfer.

Having established that the sCARfRGD adapter demonstrates significant ability to increase Ad binding to CAR-deficient breast cancer cells, we investigated its ability to improve viral infection via CAR-independent pathway. To test the utility of sCAR-ligand proteins for Ad targeting, we used both sCARfRGD and sCARfNGR proteins in Ad-mediated gene transfer assay with established human breast cancer cell lines and endothelial HUVEC cells. Our study showed that the majority of breast cancer cells are relatively refractory to Ad infection (Fig. 5). These data were corroborated by flow cytometry analysis that showed either absence or low level of CAR on their cell surface. Importantly, high levels of integrins and c-*erb*B-2 (6) were detected in these cell lines, which suggested that Ad targeting to these receptors might overcome poor vector susceptibility attributable to the lack of CAR.

To determine the magnitude of gene transfer augmentation provided by sCAR-ligand proteins they were titered against a constant dose of AdLucGFP vector (MOI 100 vp/cell) as measured by improvements in reporter gene transfer efficiency. The increase of Ad infection efficiency mediated by sCARfRGD or sCARfNGR adapters (targeted Ad) was measured by luciferase activity that was detected in infected cells compared with untargeted Ad preincubated with sCARf control protein. As shown in Fig. 6, the sCARfRGD targeting protein mediated a 3-, 4-, 6-, 8-, 11-, 13-, and 120-fold enhancement of gene transfer to BT-474, MB-468, SK-BR-3, HUVEC, AU-565, MCF-7, and MB-435 cells, respectively. Consistent with the results of binding experiments, we did not observe any significant augmentation of viral gene transfer in case of sCARfNGR. As expected, the enhanced Ad binding to cells mediated by the sCARfRGD targeting adapter correlated with augmentation of infection efficiency, as seen in gene transfer experiments. The sCARfRGD/Ad ratio providing maximal gene transfer increase ranged from $1x10^{-7}$ to $3x10^{-7}$ pmol/vp for most cell lines tested.

The magnitude of gene transfer augmentation by c-*erb*B-2-targeted Ad complexed with sCARfC6.5 adapter was illustrated on selected c-*erb*B-2-positive cell lines and c-*erb*B-2- negative MDA-MB-468 cells (6). It was shown that sCARfC6.5 targeting protein mediated a 3.4-, 11-, 32-, 47-, and 135-fold enhancement of gene transfer to MCF-7, SK-OV-3, BT-474, SK-BR-3, and AU-565 cells, respectively. Consistent with the augmentation of the Ad gene transfer to c-*erb*B-2-positive cell lines achieved by sCARfC6.5 targeting protein, sCARf control protein caused a marked decrease in Ad gene transfer. Importantly, the use of both targeting and control protein for Ad infection of c-*erb*B-2-negative MDA-MB-468 cells that express moderate levels of CAR resulted in an 8-fold decrease of gene transfer. These

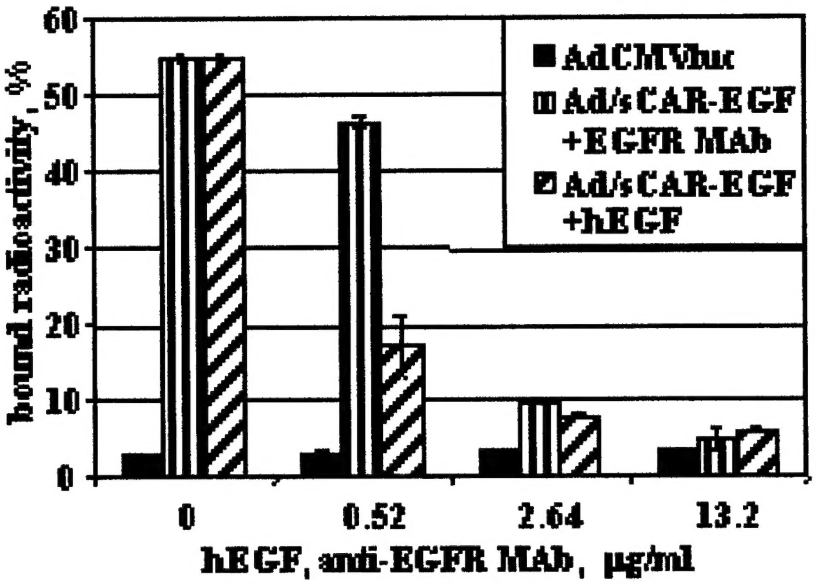

Fig. 4. Specific inhibition of sCAR-EGF-mediated Ad binding. ^{3}H-labeled Ad was preincubated for 30 min at room temperature with 0.4 mg of sCAR-EGF. Human epidermoid carcinoma A-431 cells, overexpressing EGFR, were preincubated for 30 min at 4°C in the presence or absence of either human EGF or anti-EGFR antibody at different concentrations (0.52 to 13.2 mg/ml). ^{3}H-Ad/sCAR-EGF samples (10^{5} cpm) were then added and allowed to bind for 1 h at 4°C. Cells were washed by centrifugation, and radioactivities of cell pellets were determined in a beta counter. Data are presented as the percentage of input ^{3}H-Ad bound after washing and calculated as the cumulative mean ±SD of triplicate determinations.

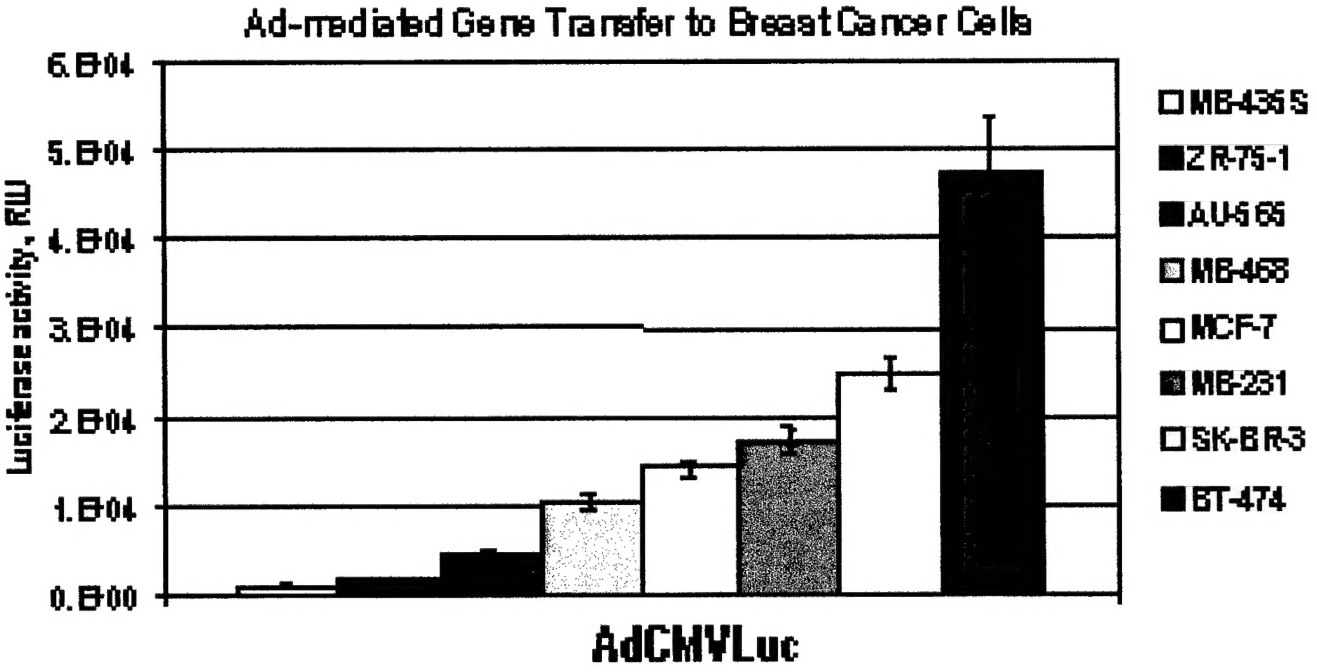

Fig. 5. Ad-mediated gene transfer to breast cancer cells. The breast cancer cell lines MDA-MB-435S, ZR-75-1, AU-565, MDA-MB-468, MCF-7, MB-231, SK-BR-3, and BT-474 were infected with AdLucGFP vector expressing luciferase reporter gene. Cells were incubated for 40 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as relative light units (*RLU*) detected in the cells. Each point represents the cumulative mean ±SD of triplicate determinations.

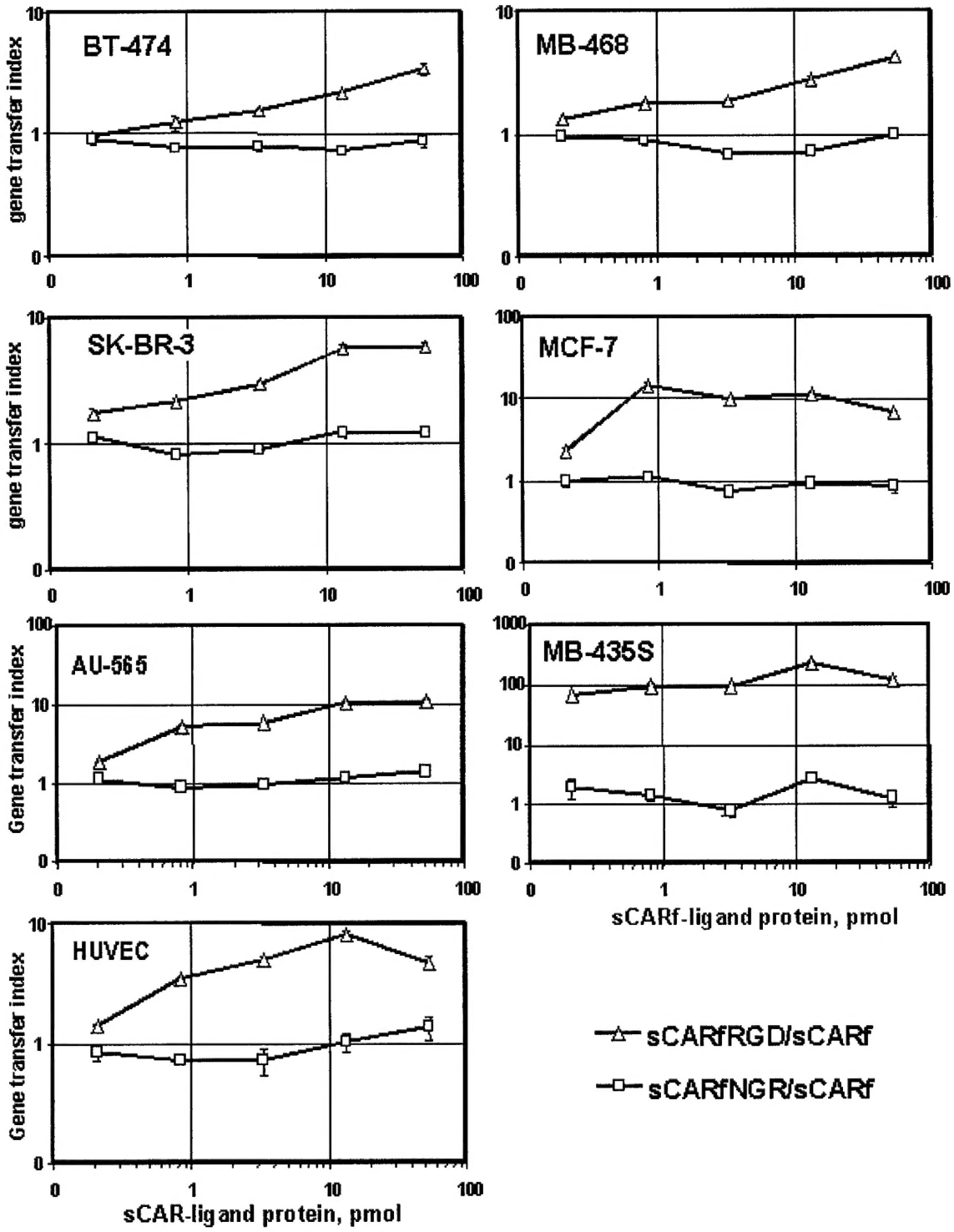

Fig. 6. Determination of optimal sCAR-ligand protein:Ad ratio. AdLucGFP vector expressing luciferase reporter gene was complexed with sCARfRGD or sCARfNGR targeting proteins or sCARf control protein at varying concentrations to form integrin-targeted, APN-targeted, or untargeted viral complexes, respectively. Monolayers of BT-474, MDA-MB-468, SK-BR-3, MCF-7, AU-565, MDA-MB-435S, and HUVEC cells were infected with targeted or untargeted viral complexes at a MOI of 100 vp/cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was measured. Results are presented as gene transfer indexes that were calculated as the ratio of luciferase activities detected in the cells infected with targeted Ad to luciferase activities detected in the cells infected with untargeted Ad complexes formed at the same concentration of each sCAR protein (*Targeted Ad:Untargeted Ad*). Each point represents the cumulative mean ±SD of triplicate determinations. Some error bars depicting SDs are smaller than the symbols.

data clearly indicate that the sCARfC6.5-targeting adapter promoted Ad infection of CAR-deficient cells specifically via a c-*erb*B-2-dependent pathway.

Validation of specificity of sCAR-ligand-mediated Ad gene transfer by blocking with free ligand or specific anti-receptor antibodies in an infection-inhibition assay.

Our primary goal is to achieve Ad targeting to breast cancer cells through receptors alternative to CAR. In this regard, the level of c-*erb*B-2-targeted viral infection could be determined by blocking Ad/sCARfC6.5 infection with a specific competitor. As was observed previously, exceeding the optimal sCAR-ligand/virus ratio proved to be inhibitory to gene transfer, apparently because of competition for cellular receptors by free sCAR-ligand protein.

To validate that sCARfC6.5-mediated virus-cell interactions occur specifically via c-*erb*B-2 we carried out an infection-inhibition assay using C6.5 scFv against c-*erb*B-2 as a competitor. Varying amounts of sCARfC6.5 were used to form Ad/sCARfC6.5 complexes prior to infection of AU-565 cells in the presence or absence of C6.5 scFv. As shown in Fig. 7, Ad gene transfer in the presence of competitor was increased 2.2-fold compared to Ad alone. In contrast, the augmentations of Ad gene transfer achieved by increasing amounts of sCARfC6.5 protein were blocked to the level of Ad alone by the presence of free C6.5 scFv. This result indicates that increases in Ad gene transfer efficiency mediated by sCARfC6.5 targeting protein occur through specific interaction with the c-*erb*B-2 oncoprotein.

In order to further validate the specificity of Ad targeting using sCAR-ligand adapter proteins we carried out an infection assay in a mixed cell culture. Ad vector expressing red fluorescent protein was preincubated with increasing amounts of sCARfC6.5 or PBS as a control and then used to infect the cell monolayers formed by Hela and AU-565 breast cancer cells. Importantly, AU-565 cells were shown to have high c-*erb*B-2 expression and absence of CAR while Hela cells express high level of CAR and no c-*erb*B-2. Infected cells were harested 24 hours post-infection and were stained for c-*erb*B-2 using indirect immunofluorescence assay in a parallel with cell mixtures infected with Ad alone and uninfected cells. Stained cell samples were then analyzed by flow cytometry to determine the percentages of c-*erb*B-2-positive (AU-565) and c-*erb*B-2-negative (Hela) cells expressing red fluorescent protein in infected and uninfected mixed cell cultures. The results of this experiment presented in Fig. 8 demonstrate that the sCARfC6.5 targeting adapter provided a 2.7- to 3-fold increase of Ad gene transfer to AU-565 cells compared to Ad alone while simultaneously decreasing gene transfer to Hela cells 18- to 27-fold. These data indicate that Ad targeting mediated by sCARfC6.5 adapter protein could improve overall selectivity of Ad infection for c-*erb*B-2-expressing breast cancer cells up to 72-fold compared to CAR-positive Hela cells.

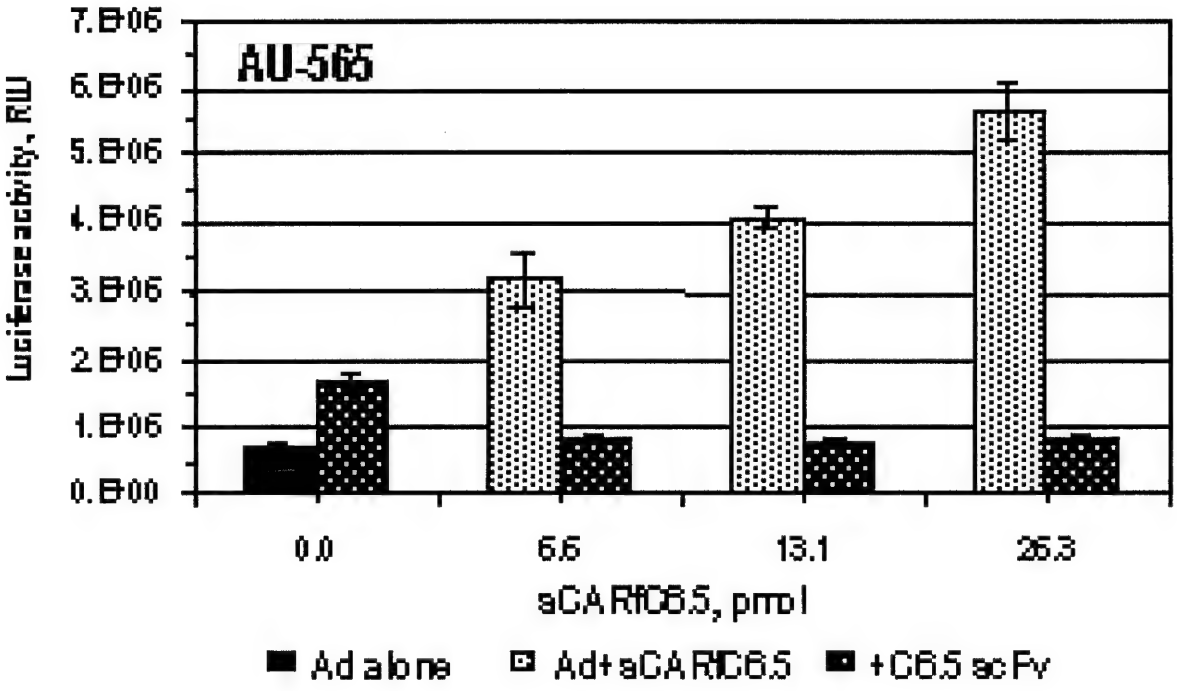

Fig. 7. Specific inhibition of sCARfC6.5-mediated Ad gene transfer. AdLucGFP vector expressing luciferase reporter gene was preincubated with PBS (*Ad alone*) or sCARfC6.5 targeting protein at varying concentrations to form c-*erb*B-2-targeted viral complexes. The monolayers of AU-565 were preincubated for 15 min at 25°C in the presence or absence of C6.5 scFv (5 μg/well) and then were infected with either targeted viral complexes or Ad alone at MOI of 100 vp/cell for 45 min at 25°C. Cells were incubated for 40 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as relative light units (*RLU*) detected in the cells infected in the presence or absence of C6.5 scFv. Each point represents the cumulative mean ±SD of triplicate determinations.

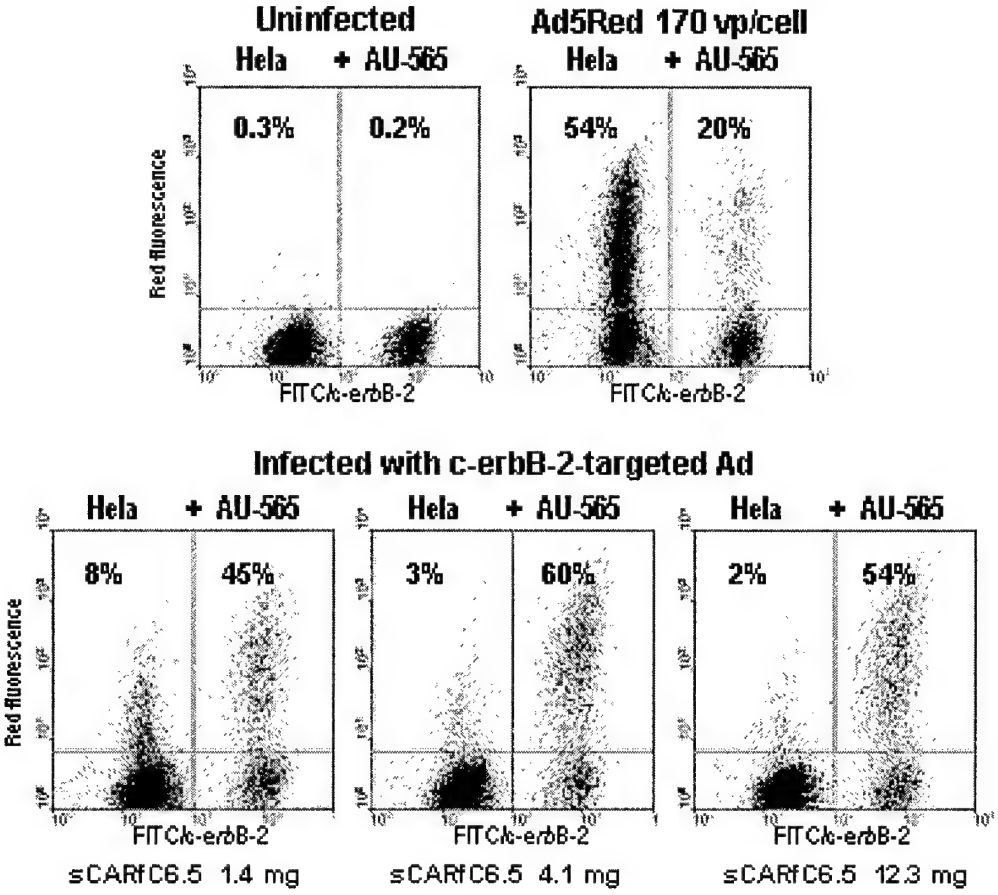

Fig. 8. Ad infection mediated by sCARfC6.5 adapter in a mixed cell culture. Ad5Red vector expressing red fluorescent protein was preincubated with 1.4, 4.1, or 12.3 μg of sCARfC6.5 (*c-erbB-2-targeted Ad*) or PBS (*Ad5Red*) and used to infect the cell monolayers formed by Hela and AU-565 cells at MOI of 170 vp/cell. Infected cells were harvested 24-h post-infection, stained for c-*erb*B-2 with Ab-2(9G6.10) mouse MAb using indirect immunofluorescence assay, and were then analysed by flow cytometry to determine the percentages of c-*erb*B-2-negative (*Hela*) and c-*erb*B-2-positive (*AU-565*) cells expressing red fluorescent protein relative to uninfected cells. The percentages of infected red fluorescent cells were calculated separately for c-*erb*B-2-negative and c-*erb*B-2-positive cell populations.

KEY RESEARCH ACCOMPLISHMENTS

- We characterized recombinant sCARfNGR, sCARfRGD, and sCARfC6.5 targeting proteins for their ability to mediate Ad binding to breast cancer and endothelial cells expressing APN, α_v-integrins, and c-*erb*B-2 oncoprotein, respectively.
- In contrast to sCARfNGR, both sCARfRGD and sCARfC6.5 adapter proteins were capable of mediating viral infection via α_v-integrins and the c-*erb*B-2 oncoprotein providing up to 120-fold increases in Ad gene transfer to breast cancer cells compared to the sCARf control protein.
- We demonstrated that targeting of an Ad vector using the sCARfC6.5 adapter in mixed cell culture improves the selectivity of Ad infection for c-*erb*B-2-positive breast cancer cells up to 72-fold compared to CAR-positive Hela cells.

REPORTABLE OUTCOMES

The following manuscripts were published:

1. Hemminki, A., Dmitriev, I., Liu, B., Desmond, R. A., Alemany, R., and Curiel, D. T. Targeting oncolytic adenoviral agents to the epidermal growth factor pathway with a secretory fusion molecule, Cancer Res. *61:* 6377-81, 2001.
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3. Pereboev, A. V., Asiedu, C. K., Kawakami, Y., Dong, S. S., Blackwell, J. L., Kashentseva, E. A., Triozzi, P. L., Aldrich, W. L., Curiel, D. T., Thomas, J. M., and and Dmitriev, I. P. Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells, Gene Ther. *9:* In press, 2002.

CONCLUSIONS

In conclusion, the work presented in this report completely addressed task 2 outlined in the approved Statement of Work. The utility of sCAR-ligand adapter proteins for Ad targeting to breast cancer cell types in vitro was characterized using virus-cell binding and gene transfer assays. The use of sCARfRGD and sCARfC6.5 targeting adapters allowed CAR-independent Ad binding and infection of breast cancer cells via α_v-integrins and the c-*erb*B-2 oncoprotein, respectively. It was shown that sCARfRGD and sCARfC6.5 adapter proteins were capable of providing up to 120-fold increases in Ad gene transfer to breast cancer cells compared to sCARf control protein. In contrast, sCARfNGR protein failed to improve Ad binding and infection of APN-positive breast and endothelial cells. This observation is inconsistent with published data regarding targeting ability of the NGR-containing peptides. The inability of the NGR peptide to target APN-expressing cells could be explained by alteration of the receptor binding properties in the context of sCAR-ligand fusion compared to phage-displayed (5) or free peptide (3, 7). To illustrate the targeting potential of sCAR-ligand approach in vitro we carried out sCARfC6.5-mediated Ad infection in heterogeneous cell populations. We demonstrated that the use of the c-erbB-2-targeting adapter in mixed cell culture improved the selectivity of Ad infection for c-erbB-2-positive breast cancer cells up to 72-fold compared to CAR-positive Hela cells.

In order to extend the utility of this Ad targeting strategy we explored the ability of sCAR-ligand adapter to enhance an oncolytic effect of replication-competent vectors. We constructed a novel replication-deficient Ad expressing a secretory sCAR-EGF adapter capable of virus retargeting to EGFR, resulting in a more than 150-fold increase in gene transfer (8). Coinfection of this vector secreting the sCAR-EGF adapter with replication-competent Ad resulted in increased oncolysis *in vitro* and therapeutic benefit *in vivo*.

These results suggest that targeting Ad vectors using recombinant sCAR-ligand adapter proteins may augment the selectivity of Ad-mediated gene transfer for breast tumors. Our data also indicate that the RGD motif, EGF, and anti-c-*erb*B-2 scFv are promising ligands for targeted Ad vector delivery to breast cancer cell types. Ad targeting approaches employing sCAR-ligand adapters may increase the efficiency of therapeutic gene delivery and decrease the toxicity of Ad vectors, which would improve the therapeutic index of cytotoxic gene therapy for carcinoma of the breast in clinical trials.

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[CANCER RESEARCH 61, 6377–6381, September 1, 2001]

Advances in Brief

Targeting Oncolytic Adenoviral Agents to the Epidermal Growth Factor Pathway with a Secretory Fusion Molecule[1]

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Abstract

Cancer gene therapy with conditionally replicating adenoviruses is a powerful way of overcoming low tumor transduction. However, one of the main remaining obstacles is the highly variable level of the coxsackie-adenovirus receptor expression on human primary cancers. In contrast, the epidermal growth factor receptor (EGFR) is overexpressed in various tumor types, and its expression correlates with metastatic behavior and poor prognosis. We constructed an adenovirus expressing a secretory adaptor capable of retargeting adenovirus to EGFR, resulting in a more than 150-fold increase in gene transfer. A replication-competent dual-virus system secreting the adaptor displayed increased oncolytic potency *in vitro* and therapeutic gain *in vivo*. This approach could translate into increased efficacy and specificity in the treatment of EGFR overexpressing human cancers.

Introduction

CRADs[3] are a promising and novel way of overcoming low tumor transduction, which is the main obstacle preventing effective gene transfer and therapeutic effect in clinical applications of cancer gene therapy (1). However, one of the main reasons why the unparalleled transduction efficacy of Ads has not translated into similar results in humans is the variable level of the CAR on primary cancers (2–9) *in vivo*. CAR is ubiquitously expressed on normal epithelial tissues and is the main receptor mediating binding of the most commonly used Ad serotypes 2 and 5. Expression of CAR may be the major factor determining the rate of transduction (4, 6, 9–11). Importantly, recent evidence (11) suggests that CAR expression may inversely correlate with the malignant potential of tumors, resulting in low infectivity of highly aggressive tumors. In contrast to the expression profile of CAR, EGFR, the prototype of cancer-associated receptors, is commonly overexpressed in many if not most carcinomas with correlation to metastatic behavior and poor prognosis (12). A powerful approach for increasing tumor transduction could be combining the tissue-penetrating capability of CRADs with the transductional control provided by retargeting moieties. In support of this hypothesis, an artificial receptor system has been used to demonstrate that the effect of Ad dispersion and subsequent oncolysis critically depends on receptor expression (13). We have constructed a novel virus that mediates secretion of a fusion molecule consisting of the extracellular domain of CAR and EGF. We then explored the capability of the sCAR-EGF to retarget Ad to EGFR. Finally, we demonstrated that infection of cancer cells with a sCAR-EGF-retargeted replication-competent dual-virus system resulted in increased oncolysis *in vitro* and a therapeutic benefit *in vivo*.

Materials and Methods

Viruses. For construction of AdsCAR-EGF, a replication-deficient Ad with sCAR-EGF in E1, the gene coding for sCAR-EGF was cloned from pFBshCAR-EGF (14) into pShuttle-CMV (Quantum, Montreal, Quebec, Canada). Homologous recombination with pAdEasy-1 (Quantum) was performed in *Escherichia coli*, followed by confirmation of structure with *Eco*RV and *Pac*I digestions, PCR, and sequencing of the transgene (data not shown). The viral genome was transfected into 293 cells for plaque purification, followed by cesium chloride purification and standard titering with OD260 and plaque assay. Resulting titers were 3.8×10^{11} VPs/ml and 1.0×10^{10} plaque-forming units/ml, ratio = 38.4 VPs/plaque-forming unit. Large-scale preparations of AdCMVLuc (a nonreplicating Ad-expressing luciferase; courtesy of Dr. Robert Gerard, Texas Southwestern Medical Center, Dallas, TX), AdsCAR6H (a nonreplicating Ad-mediating secretion of sCAR6H),[4] and Δ24 (an Ad with a 24-bp deletion in *E1A*, allowing selective replication in cells mutant in the *Rb-p16* pathway; Ref. 15) were performed with standard methods on 293 cells (or A549 cells for Δ24).

CRAdsCAR-EGF denotes a replication-competent, sCAR-EGF-secreting dual-virus system consisting of equal VPs of AdsCAR-EGF and Δ24 mixed immediately before administration to cells. CRAdCMVLuc is the respective combination of Δ24 and AdCMVLuc. Δ24 has been characterized previously (15). Validating the dual-virus strategy, it has been demonstrated that transcomplementation of E1 proteins from a plasmid or replication-competent virus results in replication of E1-deleted viruses present in the same cell (9, 16–18).

Cell Lines. 293 cells were purchased from Microbix (Toronto, Ontario, Canada). A549 (lung cancer), HeLa (cervical cancer), U118 (glioma), A431 (squamous cell skin cancer), BT474, and MB-453 (breast cancer) were obtained from the American Type Culture Collection (Rockville, MD), and SKOV3.ip1 cells (ovarian cancer) are from Dr. Janet Price (M. D. Anderson Cancer Center, Houston, TX). Cell lines were propagated in the recommended conditions.

Protein Detection. HeLa cells were infected overnight with 50 VP/cell, and BT474 and MB453 cells were infected with 500 VP/cell of AdsCAR-EGF, AdsCAR6H, and AdCMVLuc. Supernatants were collected at 48 h, and cellular debris was removed by centrifugation. Dilutions in a volume of 300 μl were transferred onto a nitrocellulose membrane using the Bio-Dot apparatus (Bio-Rad). BSA (3%) was used for blocking, followed by detection with a 1:5000 polyclonal mouse anti-CAR antibody (14) and 1:2000 goat antimouse alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) in 3% BSA. Western blot analysis on the supernatants was performed with standard methodology in a 12% two-phase gel, and proteins were detected as above. Baculovirus-expressed and -purified sCAR-EGF and sCAR6H (14) were used as controls.

Received 5/2/01; accepted 7/16/01.

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[1] Supported by the Damon Runyon-Walter Winchell Foundation, the Sigrid Juselius Foundation, the Emil Aaltonen Foundation, the Maud Kuistila Foundation, NCI (RO1 CA83821), U. S. Army (PC991018, DAmD 17-00-1-0115), the CapCure Foundation, and the Lustgarten Foundation. Gene transfer assays were performed in part at the University of Alabama at Birmingham Gene Therapy Center Correlative Laboratories for Human Clinical Trials.

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[3] The abbreviations used are: CRAD, conditionally replicative adenovirus; Ad, adenovirus; CAR, coxsackie-Ad receptor; EGF, epidermal growth factor; EGFR, EGF receptor; sCAR-EGF, secretory CAR-EGF fusion molecule; CMV, cytomegalovirus immediate early promoter; VP, viral particle; sCAR6H, secretory CAR with 6-histidine tag.

[4] I. Dmitriev, unpublished observations.

Retargeting Assays. SKOV3.ip1, U118, and A431 cells were infected in duplicate with AdCMVLuc preincubated for 30 min with aliquots of supernatants (see above). Twenty VP/cell were used for SKOV3.ip1, whereas 200 VP/cell were used for U118 and A431. Luciferase assay was performed 48 h later (Luciferase Assay System; Promega, Madison, WI).

Cell-killing Assays. SKOV3.ip1 cells were plated in triplicate and infected with Δ24 or AdCMVLuc for 1 h. Aliquots (25 μl) of AdsCAR-EGF or AdCMVLuc supernatant (from protein detection assays) were added each day. At 20 days, cells were fixed with formalin and stained with crystal violet.

Next, A431 and SKOV3.ip1 cells were infected with the CRAdCMVLuc or CRAdsCAR-EGF for 1.5 h. After incubating for 20 days with medium changes, the remaining cells were fixed and stained as above.

Preliminary toxicity analysis was performed by infecting A431 and SKOV3.ip1 cells with 50, 100, 200, 500, and 1000 VP/cell of AdsCAR-EGF and AdsCAR6H, followed by medium changes every 2 days for 20 days.

Animal Experiments. Initially, A431 cells were infected *ex vivo* with 50 VP/cell of CRAdsCAR-EGF for 1 h followed by a 5-h incubation. Cells were then collected and mixed with uninfected cells, and a total of 10^7 cells were injected into flanks of athymic CD-1/nu mice (Charles River Laboratories, Wilmington, MA; n = five/group). Tumor size was determined as the mean of the shortest and longest diameter (to avoid variability attributable to difficulty with estimation of height).

To compare CRAdsCAR-EGF to CRAdCMVLuc *in vivo*, s.c. tumors were established by injecting 5×10^6 A431 cell into both flanks of athymic mice (n = five/group). When tumors were ~5 mm ("day 0"), viruses were injected intratumorally in a 15-μl volume and tumors were measured as above. Each mouse was checked daily for the absence of pain or distress (19).

Statistics. Upon termination of the experiment, mean tumor size and SDs were calculated for each group of animals for each time point. The nonparametric change-point test (20) was used to show a systematic change in the pattern of observations as opposed to fluctuation attributable to chance. The mixed model (21) was used to longitudinally model the data on each tumor. The variance covariance structure was determined by using Akaike's Information Criteria (22). The Proc Mixed procedure in SAS v.6.12 (SAS Institute, Cary, NC) was used to examine the effects of group and time on tumor growth.

Results

Infection of Cells with AdsCAR-EGF Results in Secretion of sCAR-EGF. Initially, infection of high EGFR HeLa cells (23) with AdsCAR-EGF produced no evidence of secretion, whereas sCAR6H was secreted (Fig. 1). With low EGFR-expressing cells MB453 and BT474 (14, 24), secretion of sCAR-EGF was detected. The amount of protein was estimated at 110 ng/ml (75-cm^2 flask; 12 ml of medium). Western blot confirmed secretion (Fig. 1*B*). The altered migration rate of the protein in comparison with baculovirus-expressed sCAR-EGF perhaps resulted from altered charge caused by different glycosylation by insect cells. A preliminary investigation on sCAR-EGF toxicity was performed by infecting SKOV3.ip1 and A431 cells with various amounts of AdsCAR-EGF and AdsCAR6H without significant differences in cell viability (data not shown).

Secreted sCAR-EGF Mediates Retargeting of Ad to EGFR. Aliquots of supernatant from AdsCAR-EGF-infected BT474 cells were incubated with AdCMVLuc. The virus-supernatant mix was used for infection of SKOV3.ip1, U118, and A431 cells, which display moderate (SKOV3.ip1 and U118) to high (A431) EGFR expression and moderate (A431) to low (SKOV3.ip1 and U118) CAR expression (4, 14). A supernatant dose-dependent increase in luciferase expression was seen, with the highest readings 17.1-, 20.2-, and 158-fold higher than without retargeting for SKOV3.ip1, U118, and A431 cells, respectively (curves with triangles in Fig. 2).

With the highest amounts of supernatant from AdsCAR6H-infected cells, luciferase expression was reduced to 73%, 48%, and 65% (on SKOV3.ip1, U118, and A431, respectively) of the highest values for the series (curves with squares in Fig. 2). sCAR6H binds to Ad fiber but does not mediate binding to EGFR, thus modeling blockage of CAR-binding with sCAR-EGF.

Retargeting Replication-competent Ad to EGFR Results in Increased Cell Killing *in Vitro*. To validate Δ24 replication in SKOV3.ip1 cells, infections were performed at 0, 0.01, 0.1, or 1 VP/cell, and aliquots of supernatant (from BT474 cells infected with

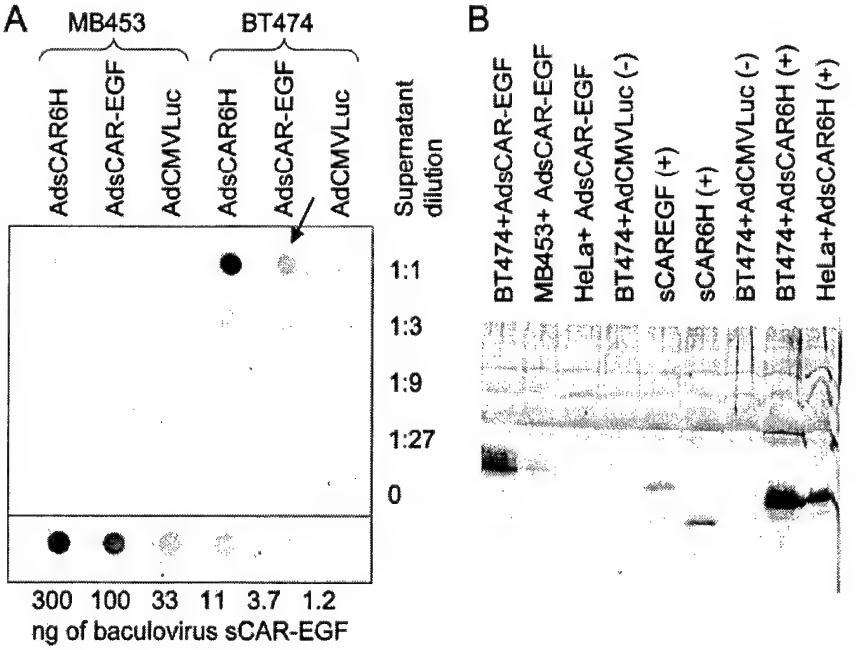

Fig. 1. Secretion of sCAR-EGF from human cancer cells infected with AdsCAR-EGF. *A*, supernatant from MB453 and BT474 (both low EGFR breast cancer lines) cells infected with AdsCAR6H (codes for human CAR ectodomain, positive control), AdsCAR-EGF, or AdCMVLuc (negative control) was centrifuged and then transferred onto a membrane. *Arrow*, the signal for sCAR-EGF. When compared with known amounts of sCAR-EGF (*lowest row*), the amount of secretion could be estimated at 110 ng/ml. *B*, Western blot suggested that the sCAR-EGF secreted from BT474 and MB453 cells (*Lanes 1–2*) was close in size to baculovirus-expressed sCAR-EGF (*Lane 5*). High EGFR HeLa cells (*Lane 3*) did not show evidence of sCAR-EGF secretion, but the positive control sCAR6H was secreted (*Lane 9*). −, supernatants collected from cells infected with AdCMVLuc (*Lanes 4 and 7*). These serve as negative controls. +, the positive controls, including *Lane 8*, which has supernatant from BT474 cells infected with AdsCAR6H. sCAR-EGF and sCAR6H (*Lanes 5–6*) are purified baculovirus-expressed proteins.

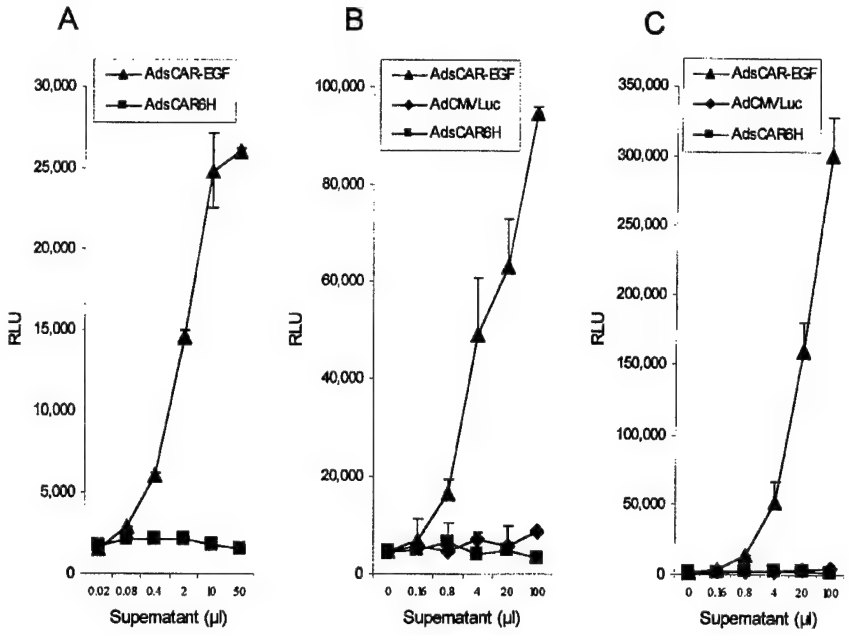

Fig. 2. Secreted sCAR-EGF mediates retargeting of Ad to EGFR. Increasing amounts of supernatant collected from cells infected with AdsCAR-EGF (curves with ▲) were incubated with AdCMVLuc, and then the mix was added to (*A*) SKOV3.ip1 (ovarian cancer), (*B*) U118 (glioma), or (*C*) A431 (squamous cell skin cancer) cells. These cells express moderate to high EGFR and thus resemble many aggressive human cancers. Supernatant from cells infected with AdCMVLuc (◆) or AdsCAR6H (■) were used as controls. Relative light units (*RLU*) are means of duplicate experiments (±1 SD). With the highest amounts of supernatant, luciferase readings were 17.1-fold (*A*), 20.2-fold (*B*), and 158-fold (*C*) higher with retargeting. The slope of the AdsCAR-EGF curves suggests that maximum retargeting potential was not reached.

AdsCAR-EGF or AdCMVLuc) were added daily. At 20 days, cell killing and partial loss of monolayer was seen only with cells that had been infected with 1 VP/cell and subjected to AdsCAR-EGF/BT474 supernatant (data not shown).

To study the effect of continuous sCAR-EGF secretion on the oncolytic potential of CRADs, we infected SKOV3.ip1 and A431 cells with the CRAdsCAR-EGF and CRAdCMVLuc dual-virus systems. On both cell lines, infection with CRAdsCAR-EGF resulted in cell killing with one to two orders of magnitude less virus than with CRAdCMVLuc (Fig. 3).

Targeting Replicative Ad to EGFR Results in a Therapeutic Advantage *in Vivo*. Various proportions of infected and uninfected A431 cells were mixed and injected s.c. (Fig. 4*A*). One percent of infected cells was sufficient to inhibit tumor growth, and 5% or more resulted in healing of tumors. None of the mice showed signs of illness or distress, suggesting that the secretion of sCAR-EGF did not cause overt toxicity.

To evaluate sCAR-EGF retargeting *in vivo*, CRAdsCAR-EGF or CRAdCMVLuc were administered with a single intratumoral injection into established A431 tumors (Fig. 4, *B–C*). The change-point test (20) revealed that the tumor growth pattern changed at 13 days for 10^9 VP CRAdsCAR-EGF ($P = 0.0045$), 21 days for 10^9 VP CRAdCMVLuc ($P = 0.0012$), 17 days for 10^8 VP CRAdsCAR-EGF ($P = 0.0026$), and 25 days for 10^8 VP CRAdCMVLuc ($P = 0.0011$), *i.e.*, 8 days earlier for CRAdsCAR-EGF with both doses.

The change-point test and the test of fixed effects (22) showed that there was a significant correlation between observations of tumor size and time ($P < 0.0001$ for all of the groups). A polynomial equation was fit for each group, thereby creating a mathematical model for each growth pattern ("modeled" in Fig. 4). The mixed model (22) was used

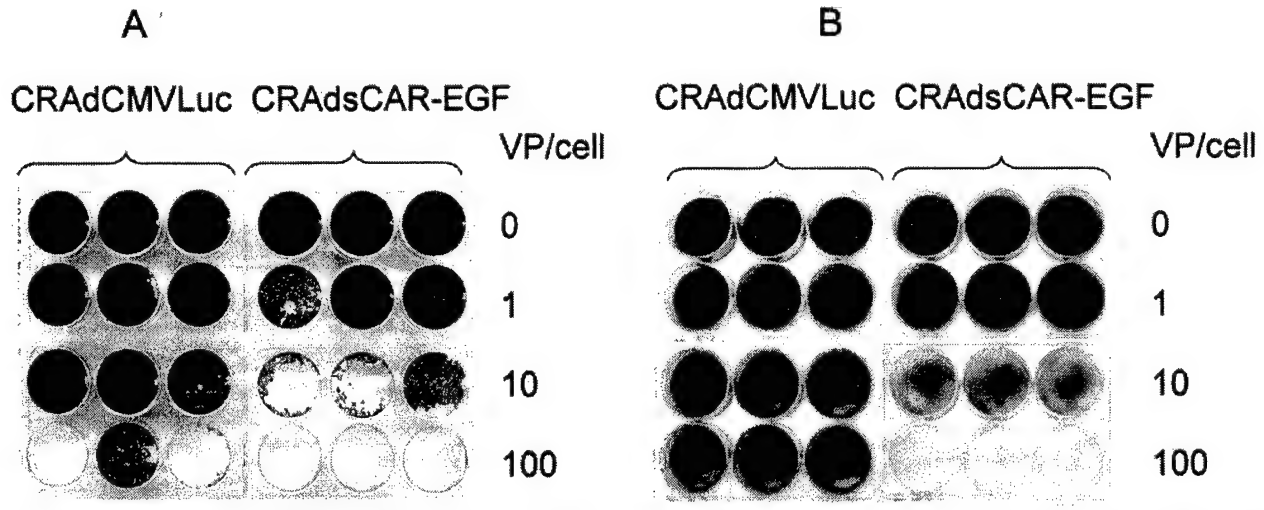

Fig. 3. Targeting oncolytic Ad to EGFR with sCAR-EGF results in an increased oncolytic effect. (*A*) SKOV3.ip1 or (*B*) A431 cells were infected with CRAdsCAR-EGF, a replication-competent sCAR-EGF-secreting dual-virus system. Oncolytic potency was compared with CRAdCMVLuc, which is isogenic in regard to replicativity but does not secrete a retargeting molecule. A similar effect on cells was observed with 1 VP/cell of CRAdsCAR-EGF as with 100 VP/cell of CRAdCMVLuc, suggesting increased oncolysis because of sCAR-EGF secretion-mediated EGFR targeting.

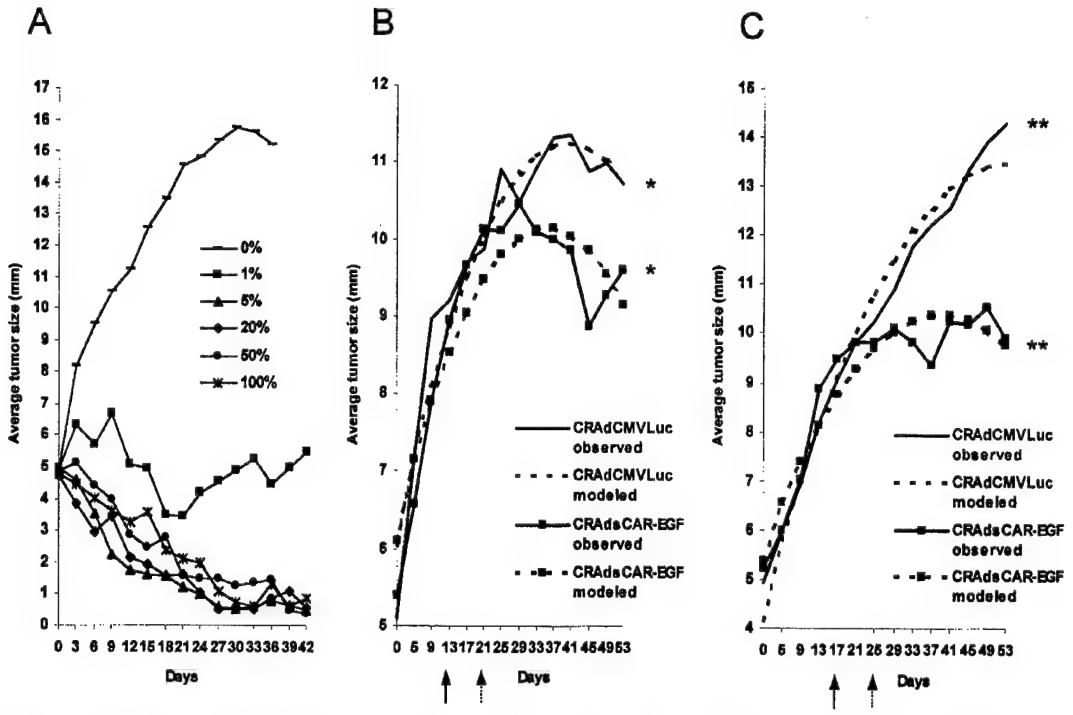

Fig. 4. sCAR-EGF secretion results in therapeutic efficacy *in vivo*. *A*, various percentages of A431 cells were infected *ex vivo* with CRAdsCAR-EGF and mixed with uninfected cells. One percent of infected cells was sufficient to inhibit tumor growth, whereas 5% or more resulted in healing of mice. Single intratumoral injections of (*B*) 10^9 or (*C*) 10^8 VP of CRAdsCAR-EGF or CRAdCMVLuc dual-virus systems were performed into established A431 xenografts. *Arrows* (*solid* for CRAdsCAR-EGF, *dotted* for CRAdCMVLuc) indicate the change-point in tumor growth characteristics, which represents the time point when oncolysis changes the initial pattern of tumor growth. On the basis of tumor size measurements (*observed*), the growth patterns were mathematically modeled for statistical comparison (*modeled*), and CRAdsCAR-EGF was found to be more oncolytic. *, $P < 0.05$; **, $P < 0.0001$.

to allow for the curvature of the plots, which was caused by the opposite effects of oncolysis and cell division. For the mice that received injections with 10^9 VP, groups were significantly different from day 29 onwards ($P < 0.05$; Fig. 4*B*). When 10^8 VP was used (Fig. 4*C*), the differences were significant from day 25 onwards ($P = 0.0066$ and 0.0003 on days 25 and 29, respectively, and < 0.0001 on days 33–53).

Discussion

In this study, we report construction of the first human Ad secreting a paracrine adaptor molecule. Secretion of sCAR-EGF was demonstrated with low EGFR cells (*Lanes 1 and 2*, Fig. 1), but not with high EGFR HeLa cells (23). In contrast, secretion was detected when HeLa cells were infected with AdsCAR6H, which codes for ectodomain of CAR but not EGF (*Lane 9*, Fig. 1*B*). EGF exhibits high affinity binding to EGFR, which leads to rapid internalization but no recycling of the receptor-ligand complex (12). Thus, perhaps secreted sCAR-EGF also internalizes. Alternatively, binding without internalization would also limit the amount of sCAR-EGF in the supernatant.

Supporting the capacity of sCAR-EGF to mediate binding and subsequent internalization of Ad, supernatant containing the fusion molecule resulted in dose-dependent increases in marker gene expression (Fig. 2). The shape of the curves suggests that the upper limit of retargeting potential was not reached. In an *in vitro* system, it is difficult to assess the capability of a secreted fusion molecule to block fiber-CAR interaction, because in the absence of CAR binding, uptake of Ad into cells can also occur via alternative mechanisms (10). However, we observed up to 52% reduction in luciferase expression with sCAR6H, which could translate into partial blocking of CAR-mediated internalization (into normal cells) by sCAR-EGF *in vivo*, but additional studies are needed.

We used a dual-virus system (CRAdsCAR-EGF) to evaluate the combination of oncolysis and EGFR targeting and saw dramatically increased killing of cells relatively low in CAR but high in EGFR expression, a combination commonly seen with primary cancer cells (Fig. 3). *In vitro*, the isogenic control virus (CRAdCMVLuc) is expected to enter cells even if they are low in CAR (10). The observed difference in oncolysis may result from more rapid internalization of the retargeted virus because of a higher number of receptors. In a living organism, extracellular viruses are at risk for neutralization by immune defenses or being swept away into organs responsible for Ad clearance. Thus, the advantage of rapid binding and internalization could be more pronounced.

s.c. xenografts are a stringent model for testing an oncolytic effect, because viral replication is balanced against rapid tumor growth. Here, we demonstrated significantly improved therapeutic efficacy of CRAdsCAR-EGF in comparison with the isogenic control not secreting sCAR-EGF (Fig. 4).

No signs of sCAR-EGF-causing toxicity were evident when cells were infected with AdsCAR-EGF in comparison with AdsCAR6H. When sCAR-EGF was added daily to SKOV3.ip1 cells infected with a CRAD, no evidence of toxicity to cells was seen. Moreover, obvious signs of toxicity were absent in mice whose xenografts were infected with CRAdsCAR-EGF. Additional studies will show whether the adaptor molecule has an effect of cell growth or whether there is toxicity *in vivo*. Also, it remains to be seen whether sCAR-EGF mediates Ad internalization via the EGFR pathway or merely substitutes for CAR in binding Ad for the native entry mechanism via penton base arginine-glycine-aspartic acid and cellular integrins.

This is the first report of a retargeting molecule secretory from human cells, but this strategy could be feasible with various high-affinity, cancer-specific ligands. Because rapid screening methods allow recognition of large numbers of cancer-specific features, unlimited possibilities for retargeting with secretory-targeting moieties

may soon be available. The dual-virus system used here provides a useful model for rendering AdsCAR-EGF replicative and investigating the combination of oncolysis and retargeting, but efficacy could be improved when *sCAR-EGF* is genetically incorporated into a CRAD.

In conclusion, we show that retargeting of replicating Ad to a receptor overexpressed in cancers is a powerful way of increasing tumor transduction and allows overcoming the lack of the primary Ad receptor. Clinical translation of this approach may be effective in treatment of a variety of human cancers that overexpress EGFR.

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[CANCER RESEARCH 62, 609–616, January 15, 2002]

Adenovirus Targeting to c-*erb*B-2 Oncoprotein by Single-Chain Antibody Fused to Trimeric Form of Adenovirus Receptor Ectodomain[1]

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ABSTRACT

The use of adenovirus (Ad) vectors for cancer gene therapy applications is currently limited by several factors, including broad Ad tropism associated with the widespread expression of *c*oxsackievirus and *a*denovirus *r*eceptor (CAR) in normal human tissues, as well as limited levels of CAR in tumor cells. To target Ad to relevant cell types, we have proposed using soluble CAR (sCAR) ectodomain fused with a ligand to block CAR-dependent native tropism and to simultaneously achieve infection through a novel receptor overexpressed in target cells. To confer Ad targeting capability on cancer cells expressing the c-*erb*B-2/HER-2/neu oncogene, we engineered a bispecific adapter protein, sCARfC6.5, that consisted of sCAR, phage T4 fibritin polypeptide, and C6.5 single-chain fragment variable (scFv) against c-*erb*B-2 oncoprotein. Incorporation of fibritin polypeptide provided trimerization of sCAR fusion proteins that, compared with monomeric sCAR protein, resulted in augmented affinity to Ad fiber knob domain and in increased ability to block CAR-dependent Ad infection. We demonstrated that sCARfC6.5 protein binds to cellular c-*erb*B-2 oncoprotein and mediates efficient Ad targeting via a CAR-independent pathway. As illustrated in cancer cell lines that overexpress c-*erb*B-2, targeted Ad, complexed with sCARfC6.5 adapter protein, provided from 1.5- to 17-fold enhancement of gene transfer compared with Ad alone and up to 130-fold increase in comparison with untargeted Ad complexed with sCARf control protein. The use of recombinant trimeric sCAR-scFv adapter proteins may augment Ad vector potency for targeting cancer cell types.

INTRODUCTION

Ad[3] represents a large family of nonenveloped viruses (1). Human Ad includes 47 known viral serotypes grouped into six distinct subgroups, A to F. Most of the studies on the mechanism of Ad infection have concluded that receptor recognition is one of the key factors that determines cell tropism (2, 3). In this regard, the initial steps of Ad infection involve at least two sequential virus-cell interactions, each mediated by a specific viral capsid protein. Ad infection is initiated by the binding of globular knob domain of trimeric fiber protein to a host cell primary receptor (4, 5). Subsequent interaction of the penton base with α_v integrins mediates virion internalization via receptor-mediated endocytosis (6). Fiber receptor for Ad subgroups A, C, D, E, and F has been identified as the CAR (7–9). CAR is an integral membrane protein consisting of two extracellular immunoglobulin-like D1 and D2 domains, a transmembrane region, and a COOH-terminal cytoplasmic domain (8, 10). The extracellular domain of CAR is sufficient for virus attachment and infection (11, 12), whereas both transmembrane and intracellular regions appear to be dispensable for these functions (13). Both structural analysis of fiber knob complexed with CAR D1 domain (14) and knob mutagenesis studies (15) revealed that amino acid residues responsible for CAR binding are located on lateral surfaces formed by the interface of two adjacent knob monomers. These data suggest an avidity mechanism when three CAR molecules could simultaneously bind per one fiber knob trimer, which was recently supported by kinetic analysis of Ad2 knob binding to the CAR D1 domain (16).

Well-characterized Ad serotypes 2 and 5 from subgroup C are predominantly used as vectors for *in vitro* and *in vivo* gene delivery (17), because of high infection efficiency in a variety of human cell types and tissues. However, this broad viral tropism is disadvantageous for gene delivery to cancer cell types refractory to Ad infection because of the absence or low levels of CAR expression (18–21). This limitation could be solved by Ad targeting via a nonnative viral receptor (22, 23). Several strategies have been tested in an effort to target Ad via CAR-independent pathways (24) including chemical conjugation or genetic modification of viral capsid proteins to incorporate targeting ligands and the use of bispecific adapter molecules to provide indirect virus linkage with the cell-surface receptors. The technical achievement of Ad targeting via adapter molecules has been approached by a variety of methods. Bispecific conjugates of antibodies or their Fab fragments were used to achieve linkage between target receptor and v.p. by means of specific recognition through either a fiber knob domain or penton base (reviewed in Refs. 17, 22, 23). Further refinement of this strategy has been accomplished by the engineering of recombinant proteins consisting of an anti-knob scFv fused with human EGF (25) or a scFv against EGFR (26). The original concept of employment of fusion proteins comprising a soluble viral receptor and targeting ligand was proposed for retrovirus targeting to specific cell types (27). Applying this strategy to Ad targeting, we have developed an approach based on the use of sCAR ectodomain fused with EGF, achieving simultaneously the blocking of virus-CAR interaction and the redirection of Ad to cells overexpressing EGFR (28, 29). A similar approach was successfully applied to target Ad to high-affinity Fcγ receptor I-positive human monocytic cells (30). The use of recombinant adapter molecules eliminates chemical conjugation and provides a high degree of flexibility for ligand substitution and, consequently, expands the targeting capabilities of Ad vectors.

We hypothesized that the predicted 3:1 stoichiometry of CAR-knob binding could provide high-affinity linkage of trimeric sCAR-ligand proteins to v.p. and, thereby, promote the ligand-mediated binding to target receptors. In this study, we describe a novel approach of Ad transductional targeting to cancer cell types expressing c-*erb*B-2 oncoprotein by means of a recombinant protein adapter. The gene known as c-*erb*B-2/HER-2/*neu*, encoding a member of the *erb*B family of growth factor receptors, is most frequently altered in human cancer and was shown overexpressed in a number of malignancies including tumors that arise in the breast and ovary (31, 32). We engineered a bispecific protein, sCARfC6.5, featuring a unique trimeric design and consisting of sCAR fused with phage T4 fibritin polypeptide and C6.5 scFv against c-*erb*B-2. We have demonstrated that the sCARfC6.5 protein efficiently blocks Ad native tropism while simultaneously

Received 8/17/01; accepted 11/14/01.

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[1] Supported by Idea Award DAMD17-00-1-0115 from United States Army Department of Defense (I. P. D.), National Cancer Institute Grant N01 C0–97110, and NIH Grants P50 CA89019 and RO1 CA86881.

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[3] The abbreviations used are: Ad, adenovirus; CAR, coxsackievirus and Ad receptor; FACS, fluorescent-activated cell sorting; sCAR, soluble CAR ectodomain; scFv, single-chain fragment variable; EGF, epidermal growth factor; EGFR, EGF receptor; MAb, monoclonal antibody; GFP, green fluorescent protein; v.p., viral particle(s); MOI, multiplicity/multiplicities of infection; FBS, fetal bovine serum.

mediating virus infection via an alternative CAR-independent pathway, which markedly enhances gene transfer efficiency to cell lines that overexpress c-*erb*B-2. Our data suggest that the use of this original approach may augment the potency of Ad vectors for cancer gene therapy.

MATERIALS AND METHODS

Cells and Media. The 293 human kidney cell line, transformed with Ad5 DNA, was purchased from Microbix (Toronto, Ontario, Canada). The human breast cancer cell lines MDA-MB-468, AU-565, SK-BR-3, BT-474, and MCF-7 and the ovarian cancer cell line SK-OV-3, established from adenocarcinomas of mammary gland and ovary, respectively, were from the American Type Culture Collection (Manassas, VA). All of the cell lines were maintained in recommended growth media supplied by Mediatech (Herndon, Va.) containing 10% FBS (HighClone, Logan, UT) and 2 mM glutamine at 37°C in a humidified atmosphere of 5% CO_2. Infection of the cells with Ad was carried out in the infection medium containing 2% FBS.

Enzymes. Restriction endonucleases, Klenow enzyme, T4 DNA ligase, and proteinase K were from either New England Biolabs (Beverly, MA) or Boehringer Mannheim (Indianapolis, IN).

Antibodies. Murine serum to baculovirus-produced human sCAR protein was generated at the University of Alabama at Birmingham Hybridoma Core Facility. The MAb RmcB (33) to human CAR were produced using hybridoma purchased from American Type Culture Collection and kindly provided by J. T. Douglas (University of Alabama at Birmingham). Penta·His MAbs were from Qiagen Inc. (Valencia, CA). Rabbit serum against phage T4 fibritin protein was kindly provided by V. Mesyanzhinov (Shemykin and Ovchinnicov Institute of Bioorganic Chemistry, Moscow, Russia). Mouse MAbs to the human c-*erb*B-2/HER-2/neu oncoprotein, Ab-2 (Clone9G6.10), were purchased from NeoMarkers Inc., (Fremont, CA). Normal mouse IgG1 were from OEM Concepts (Toms River, NJ). Goat antimouse and antirabbit IgG conjugated with alkaline phosphatase were from Sigma Chemical Co. (St Louis, MO) and Pierce (Rockford, IL), respectively. Streptavidin-alkaline phosphatase conjugate was from Bio-Rad Laboratories (Hercules, CA). Alexa 488-labeled goat antimouse IgG were from Molecular Probes (Eugene, OR).

Viruses. A recombinant Ad5 vector, AdLucGFP, containing double expression cassette consisting of firefly luciferase gene and *GFP* gene under the control of cytomegalovirus immediate early promoter in place of the E1 region of the Ad genome, was constructed as described by Seki *et al.* (34). Ad was propagated on 293 cells and purified by centrifugation in CsCl gradients by a standard protocol. The titers of physical v.p. and infectious v.p. were determined by using the methods of Maizel *et al.* (35) and Mittereder *et al.* (36), respectively.

Construction of Recombinant Plasmids. To generate the recombinant gene encoding the extracellular domain of human CAR followed by polypeptide sequence derived from bacteriophage T4 fibritin protein (37), PCR was used. Sense primer 5′-GTT GAA AGA TCT GGA TTA ACC AAT AAA ATA AAA GCT ATC GAA ACT GAT ATT GCA TCA G complementary to position 1240 of the fibritin gene was designed to introduce *Bgl*II restriction site into the amplified DNA sequence, and antisense primer 5′-TTG CGG CCC CAG CGG CCG CTG GTG ATA AAA AGG TAG complementary to position 16 of untranslated 3′-region was designed both to introduce *Not*I restriction site and to substitute a stop codon for an alanine (GCC) codon. The PCR fragment (238 bp) was digested with *Bgl*II and *Not*I, and a 214-bp DNA fragment encoding 71 COOH-terminal amino acids of fibritin M polypeptide (38) was purified. A *Bgl*II-*Not*I-fragment was ligated with *Bam*HI- and *Not*I-digested plasmid pFBshCAR-EGF (29) that contained the recombinant gene for the CAR ectodomain, His_6, short linker, and human EGF to substitute EGF for the fibritin sequence. This plasmid, designated pFBsCARfibritin, was then cleaved with *Not*I and ligated with oligonucleotide duplex 5′-GGC CCA ACC GCA GCC AAA ACC TCA ACC CCA GCC ACA ACC TCA GCC CAA ACC TCA GCC TAA ACC GGT TTA AAC GGC C coding for a proline-rich hinge region that was derived from camel immunoglobulins and containing an *Age*I site followed by a stop codon. A plasmid clone that contained the DNA duplex in the correct orientation was selected by sequencing and was designated pFBsCARfCh. The resultant plasmid was then used as a vector to generate the recombinant baculovirus using the Bac-to-Bac baculovirus construction system (Life Technologies, Inc., Grand Island, N.Y.) to express sCARf protein. Then, oligonucleotides 5′-CCG GGA GCT CTG CGC TAG CT and 5′-CCG GAG CTA GCG CAG AGC TC, designed to contain *Sac*I and *Nhe*I restriction sites and *Age*I-compatible cohesive 5′-ends, were annealed to form duplex DNA and ligated to *Age*I-digested pFBsCARfCh. Plasmid clones were sequenced, and the plasmid containing the DNA duplex in the correct orientation was designated pFBsCARfChSN. DNA sequence coding for C6.5 scFv against c-*erb*B-2 was PCR amplified from cDNA (provided by J. D. Marks, Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA) using primers 5′-AGG AAA CCG GTG GTC TAG ATC AGG TGC AGC and 5′-AGT ATC TAG AGG GAA CTA GTA CGG TCA GCT TGG TCC CTC, which were designed to introduce *Xba*I and *Spe*I restriction sites, respectively. The PCR product was digested with *Xba*I and *Spe*I, and a purified 769-bp DNA fragment was cloned into *Spe*I-cleaved pFastBacHTa (Life Technologies, Inc.), which resulted in plasmid pFB6hC6.5. Then, plasmid pFBsCARfChSN was digested with *Sac*I and *Nhe*I and ligated with 775-bp *Sac*I-*Spe*I-fragment DNA, coding for C6.5 scFv isolated from pFB6hC6.5. The constructed plasmid, containing recombinant gene encoding sCAR, His_6, short linker, fibritin polypeptide, hinge region, and C6.5 scFv, was sequenced to confirm the correct DNA structure. The resultant plasmid, designated pFBsCARfC6.5, was then used to generate the recombinant baculovirus using the Bac-to-Bac system.

Expression, Purification, and Biotinylation of the Fusion Proteins. The fusion proteins, sCARf and sCARfC6.5, comprised of sCAR-His_6-fibritin and sCAR-His_6-fibritin-hinge-C6.5scFv polypeptide sequences, respectively, were expressed in High Five cells (Invitrogen, Carlsbad, CA) that were infected with recombinant baculoviruses. Recombinant His_6-tagged proteins were purified from dialyzed culture medium by immobilized metal-ion-affinity chromatography on Ni-nitrilotriacetic acid (Ni-NTA)-Sepharose (Qiagen Inc.) as described previously (29). Protein concentrations were determined by the BCA-200 protein assay kit using bovine gamma globulin as the standard (Pierce). Purified sCARf and previously produced sCAR-His_6 (29) proteins were biotinylated using EZ-Link SulfoNHS-LS-Biotinylation kit (Pierce). The degree of biotin-protein incorporation [determined using HABA method (Pierce)] was 0.6 biotin per molecule of sCAR-His_6 monomeric protein and 0.5 biotin per trimeric molecule of sCARf protein.

Protein Electrophoresis and Western Blot. To determine whether the recombinant sCARf and sCARfC6.5 fusion proteins could form trimers, they were analyzed by SDS-PAGE. Purified proteins were either boiled in Laemmli loading buffer prior to electrophoresis to denature proteins to monomers or loaded on the gel without denaturation. The trimeric or monomeric configurations of protein molecules were determined based on their mobilities in the gel. To analyze the composition of sCAR fusion proteins, we used Western blot. Samples of boiled sCARfC6.5 and sCARf proteins separated on 4–15% gradient SDS-PAGE were transferred to polyvinylidene difluoride membrane and probed with murine anti-sCAR serum, Penta·His MAb, or rabbit antifibritin serum. Bound IgG were detected with secondary alkaline phosphatase-conjugated antibodies.

ELISA. Solid-phase binding ELISA was performed as follows. Recombinant Ad5 knob protein, expressed in *Escherichia coli* as described previously (39), was diluted in 50 mM $NaHCO_3$ (pH 9.6) at a concentration of 1 μg/ml and was immobilized on Nunc-Maxisorp ELISA plate overnight. The wells were blocked with PBS [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), and 136 mM NaCl] containing 0.05% Tween 20 and 2% BSA and then were washed with PBS containing 0.05% Tween 20. Biotinylated sCAR-His_6 and sCARf proteins, diluted in blocking buffer to concentrations ranging from 0.01 to 25 pmol/ml, were added to the wells in 100-μl aliquots. After a 1-h incubation at room temperature, the wells were washed, and bound biotinylated proteins were detected by 45-min incubation with 1:1000 dilution of streptavidin-alkaline phosphatase conjugate (Bio-Rad). The plates were then developed using signal-producing reagent *p*-nitrophenyl phosphate (Sigma Chemical Co.). Plates were read in a microtiter plate reader, set at 405 nm; results are presented as mean absorbance ± SD.

Indirect Immunofluorescence. The analysis of cell lines for expression levels of CAR and c-*erb*B-2 oncoprotein was performed by indirect immunofluorescence assay using flow cytometry as follows. Aliquots (100 μl) of cells, resuspended in FACS buffer [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), 136 mM NaCl, 1% BSA, and 0.1% NaN_3] at a concentration of 2×10^6 cells/ml were incubated with either RmcB (anti-CAR) or Ab-2 (anti-c-*erb*B-2) MAb at

a concentration of 5 μg/ml for 1 h at 4°C. An isotype-matched normal mouse IgG1 was used as a negative control. Cells were washed with FACS buffer by centrifugation and then were incubated with secondary Alexa 488-labeled goat antimouse antibody (Molecular Probes) at a concentration of 5 μg/ml for 1 h at 4°C. Cells were washed with FACS buffer prior to flow cytometry analysis. To validate that C6.5 scFv, incorporated in the context of sCARfC6.5 protein, are able to bind to cellular c-*erb*B-2, cells were incubated first with sCARfC6.5 or with sCARf protein as a negative control at a concentration of 10 μg/ml. After a 1-h incubation, cells were washed and incubated with primary RmcB MAb and then with secondary Alexa 488-labeled antibody as described above. Cell samples (10^4 cells/sample) were analyzed by flow cytometry performed at the University of Alabama at Birmingham FACS Core Facility. Data were expressed as the geometric mean fluorescence intensity of the entire gated population. The positive cell population was determined by gating the right-hand tail of the distribution of the negative control sample for each cell line at 1%.

Gene Transfer Assay. The assay of Ad-mediated gene transfer to the cells was performed as follows. Aliquots (3 μl) of AdLucGFP vector were mixed with 6-μl aliquots of sCARf or sCARfC6.5 protein dilutions ranging from 0.2 to 53 pmol or of PBS [10 mM NaH_2PO_4, 10 mM KH_2PO_4 (pH 7.4), and 136 mM NaCl] for 15 min at room temperature. The virus-sCARprotein-containing complexes were diluted to 1 ml with infection medium containing 2% FBS, and 200-μl aliquots were then added to the cell monolayers [grown in a 24-well plate ($3–5 \times 10^5$ cells/well) at MOI of 100 v.p./cell] and were incubated for 30 min at room temperature to allow virus internalization. Then, infection medium was aspirated, the cells were washed with PBS, and the cells were incubated in a growth medium containing 10% FBS at 37°C to allow expression of the reporter genes. Forty-six h postinfection, cells were lysed and luciferase activity was analyzed by using the Promega (Madison, WI) luciferase assay system and a Berthold (Gaithersburg, MD) luminometer. For inhibition of the Ad infection of 293 cells AdLucGFP vector was mixed with sCARf or sCARfC6.5 protein dilutions (1.1–30 pmol), or with sCAR-His_6 protein dilutions (3–230 pmol), or with PBS for 15 min at room temperature. Monolayers of 293 cells were exposed to the virus-sCARprotein-containing complexes at MOI of 13 v.p./cell for 30 min and then were incubated for an additional 20 h at 37°C to allow expression of luciferase gene prior to analysis.

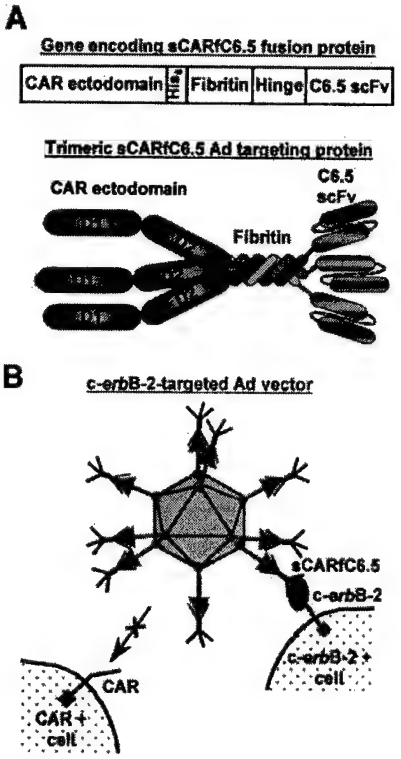

Fig. 1. *A*, design of sCARfC6.5 fusion protein. The recombinant fusion gene encoding the human CAR ectodomain, His_6, polypeptide derived from phage T4 fibritin protein (*Fibritin*); proline-rich hinge region (*Hinge*), and C6.5 scFv to c-*erb*B-2 oncoprotein was constructed in a baculovirus expression vector. The sequence encoding fibritin polypeptide was introduced into the design to achieve sCARfC6.5 protein trimerization. Recombinant gene was expressed in baculovirus-infected insect cells and secreted sCARfC6.5 protein was purified. *B*, use of sCARfC6.5 protein for Ad targeting. Engineered bispecific sCARfC6.5 fusion protein serves as an adapter between Ad and cells expressing c-*erb*B-2 oncoprotein. Presence of three sCAR domains in the context of trimerized adapter molecule potentially provides high-affinity viral linkage because of the trivalent stoichiometry of CAR-knob binding. The use of trimeric sCARfC6.5 adapter protein might, therefore, allow efficient blocking of Ad infection of CAR-bearing cells. C6.5 scFv targeting moiety of virus-bound adapter protein mediates recognition of c-*erb*B-2-positive cells, thereby providing novel target-specific Ad tropism.

RESULTS

Design and Generation of sCAR Fusion Proteins. To exploit the trivalent nature of CAR-knob interaction for the purposes of Ad targeting, we engineered a recombinant adapter protein consisting of soluble CAR in fusion with a trimerization sequence and a targeting ligand (Fig. 1*A*). We hypothesized that the predicted 3:1 stoichiometry of CAR-knob binding could provide high-affinity linkage of trimeric sCAR-ligand adapter proteins to virus and thereby block CAR-dependent Ad infection. Our goal was to generate a trimeric sCAR-ligand protein capable of efficiently blocking Ad native tropism while providing a novel target-selective tropism to c-*erb*B-2-positive cells (Fig. 1*B*). Because of the absence of a specific cognate ligand for c-*erb*B-2 oncoprotein, we chose C6.5 scFv as a targeting moiety that binds to the extracellular domain of this tumor antigen (40).

The gene encoding the extracellular part of human CAR including secretion signal, six-histidine tag (His_6), flexible linker, trimerization domain, hinge region, and C6.5 scFv was designed to produce sCARfC6.5 targeting protein. To achieve trimerization of the sCAR fusion molecule, we used a polypeptide derived from bacteriophage T4 fibritin protein, which is known to form highly stable homotrimers (37). Genetically engineered fibritin M polypeptide (38), containing 71 amino acids corresponding to the last α-helical coiled-coil segment and the complete COOH-terminal domain of phage T4 fibritin protein, was used. A proline-rich hinge region (20 amino acids) derived from camel antibodies served as a junction between the fibritin polypeptide and downstream scFv. The gene encoding sCAR, His_6, linker, fibritin M, and hinge region was used to produce sCARf control protein. The constructed genes for sCARf and sCARfC6.5 proteins were expressed in a baculovirus expression system that has already proved its utility for the expression of functional sCAR (9) and chimeric sCAR-EGF (29). Both of the fusion proteins were produced in a secreted soluble form after infection of High Five insect cells with generated recombinant baculoviruses. Secreted sCARf and sCARfC6.5 His_6-tagged proteins were purified and were analyzed for the presence of encoded polypeptide sequences and trimerization.

Characterization of Recombinant sCAR Fusion Proteins. The polypeptide composition of produced fusion proteins was characterized by Western blot analysis. Detection of denatured electrophoretically resolved sCARf and sCARfC6.5 proteins using specific antibodies revealed the presence of the CAR ectodomain, His_6 tag, and fibritin sequences in the context of both of the fusion proteins (Fig. 2*A*). The incorporation of scFv sequence in sCARfC6.5 protein was confirmed by the shift of its electrophoretic mobility compared with sCARf, indicating the predicted 27 kDa increase of molecular mass. The presence of additional minor bands in the sample of sCARfC6.5 protein was likely the result of incomplete translation of sCARfC6.5 mRNA. To determine whether the recombinant secreted sCARf and sCARfC6.5 proteins form trimers, these proteins were analyzed by SDS-PAGE. Electrophoresis of denatured protein samples showed the presence of major bands with molecular masses close to 36 and 63 kDa as expected for monomeric forms of sCARf and sCARfC6.5 molecules, respectively (Fig. 2*B*). Electrophoretic mobility of nonde-

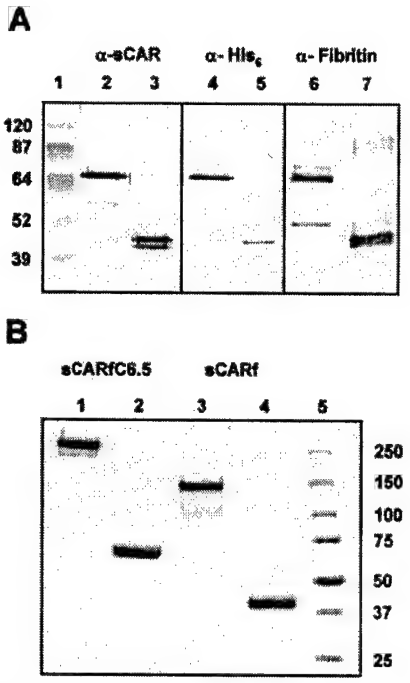

Fig. 2. Characterization of sCAR fusion proteins. *A*, Western blot of sCAR fusion proteins. Samples of purified sCARfC6.5 (*Lanes 2*, *4*, and *6*) and sCARf (*Lanes 3*, *5*, and *7*) proteins were boiled in Laemmli loading sample buffer and separated on 4–15% gradient SDS-PAGE. Electrophoretically resolved proteins were transferred to polyvinylidene difluoride membrane and probed with murine anti-sCAR serum (*Lanes 2* and *3*), Penta·His MAb (*Lanes 4* and *5*), or rabbit anti-fibritin serum (*Lanes 6* and *7*). Bound murine or rabbit IgG were detected with secondary alkaline phosphatase-conjugated goat antimouse or antirabbit antibodies, respectively. *Numbers on the left*, molecular masses of marker proteins (*Lane 1*) in kilodaltons. *B*, trimerization analysis of sCAR fusion proteins. Samples of sCARfC6.5 (*Lanes 1* and *2*) and sCARf (*Lanes 3* and *4*) proteins and molecular mass marker (*Lane 5*) were separated on 4–15% gradient SDS-PAGE. The samples in *Lanes 2* and *4* were boiled in Laemmli loading buffer to denature proteins to monomers, whereas proteins in *Lanes 1* and *3* were not denatured by boiling. Protein bands were visualized by GELCODE blue stain reagent. *Numbers on the right*, molecular masses of marker proteins in kilodaltons.

natured protein samples was greatly decreased compared with denatured proteins as was predicted for trimeric forms of sCARf and sCARfC6.5 molecules. This demonstrated that incorporation of fibritin polypeptide in the context of these recombinant fusion proteins results in efficient trimerization of both sCARfC6.5 and sCARf control protein. Thus, the analysis of sCARf and sCARfC6.5 proteins indicates that generated sCAR fusion proteins maintain both designed composition and stable trimeric conformation.

Analysis of sCAR Fusion Proteins Binding to Ad Fiber Knob. We characterized trimeric sCARf protein with respect to its ability to bind Ad fiber knob compared with monomeric sCAR-His$_6$ protein generated previously (29). The knob-binding affinities of sCARf and sCAR-His$_6$ proteins were compared by ELISA using immobilized Ad5 knob expressed in *E. coli* (39). Compared with monomeric sCAR-His$_6$ protein, the knob-binding affinity of trimeric sCARf protein was increased at least 20-fold in a range of tested concentrations from 0.1 to 5 pmol/ml (Fig. 3*A*). This result suggests that, compared with sCAR-His$_6$ monomer, trimeric sCARf protein possesses augmented ability to provide viral linkage by means of high-affinity binding to Ad fiber knob domain. Therefore, trimeric sCAR fusion proteins might offer improved blocking capability of CAR-dependent virus-cell attachment and viral infection.

To determine whether augmented binding to Ad fiber knob results in increased ability of trimeric sCAR proteins to block Ad infection, we performed an infection inhibition assay. AdLucGFP vector, expressing both luciferase and *GFP* reporter genes, was preincubated with either PBS or one of the sCAR-His$_6$, sCARf, or sCARfC6.5 proteins at varying concentrations and was used to infect 293 cells, which are known to express a high level of CAR. The ability of sCAR fusion proteins to block viral infection was assessed by sCAR protein dose-dependent impairment of Ad-mediated gene transfer as measured by luciferase activity in infected cells (Fig. 3*B*). It was shown that sCARfC6.5 as well as sCARf displayed an increased ability to inhibit CAR-dependent Ad infection compared with monomeric sCAR-His$_6$ protein. The concentrations of sCARfC6.5, sCARf, and sCAR-His$_6$ needed to block Ad infection by 50% were 3, 6, and 54 nM, respectively. Interestingly, sCARfC6.5 protein displayed Ad infection inhibition efficiency somewhat higher than did sCARf control protein. This experiment validated the utility of trimeric sCAR fusion proteins to block CAR-dependent Ad tropism and, therefore, provided a rationale for additional c-*erb*B-2 targeting studies.

Bispicific sCARfC6.5 Protein Binds to Cellular c-*erb*B-2. Flow cytometry analysis was performed to validate that C6.5 scFv incorporated into recombinant sCARfC6.5 fusion protein retained its ability to bind c-*erb*B-2 oncoprotein at the cell surface. The sCARfC6.5 protein was used to bind to c-*erb*B-2 that was overexpressed on AU-565 breast cancer cells. The MDA-MB-468 breast cancer cell line, previously shown to be c-*erb*B-2-negative, was used as a control. The sCARfC6.5 protein, bound to c-*erb*B-2 displayed at the cell

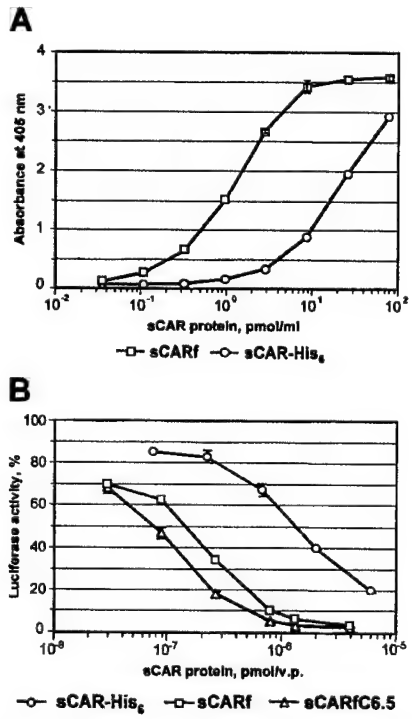

Fig. 3. Analysis of sCAR fusion proteins binding of to Ad fiber knob. *A*, comparison of monomeric and trimeric sCAR protein knob-binding by ELISA. Biotinylated trimeric sCARf and monomeric sCAR-His$_6$ fusion proteins were incubated at various concentrations with immobilized recombinant Ad5 fiber knob protein. Biotinylated sCAR fusion proteins bound to fiber knob were detected with alkaline phosphatase conjugated with streptavidin. *Each point*, the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols), SDs. *B*, inhibition of Ad infection of CAR-positive cells by sCAR fusion proteins. AdLucGFP vector containing luciferase expression cassette was incubated with either PBS or with increasing amounts of monomeric sCAR-His$_6$, or trimeric sCARf, or sCARfC6.5 fusion proteins. Viral mixtures were added to monolayers of 293 cells at MOI of 100 v.p./cell. After 30-min incubation to allow virus internalization, the medium was changed and cells were incubated for an additional 20 h at 37°C to allow luciferase expression. Then, cells were lysed, and relative luciferase activity was analyzed. Luciferase activities, detected in cells infected in the presence of sCAR fusion proteins, are shown as *percentages of luciferase activity* registered in control cells infected with AdLucGFP incubated with PBS. Each point represents the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols), SDs.

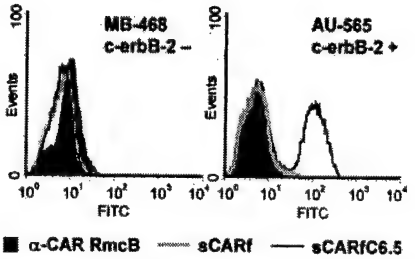

Fig. 4. Confirmation of sCARfC6.5 protein binding to cellular c-*erb*B-2. Trimeric sCARfC6.5 and sCARf fusion proteins were incubated with either c-*erb*B-2-positive AU-565 or c-*erb*B-2-nagative MB-468 cells. The sCAR fusion proteins bound to cells were probed with anti-CAR RmcB MAb and then detected with secondary Alexa 488-labeled goat antimouse antibodies. Binding of sCARfC6.5 protein (*black line*) to c-*erb*B-2-positive AU-565 cells is seen because of the positive staining relative to sCARf control protein (*gray line*) or anti-CAR MAb alone (*spike filled in black*). Representative data from two independent experiments are shown.

surface, presented the CAR ectodomain for antibody detection with primary anti-CAR RmcB MAb (33) followed by a secondary anti-mouse fluorochrome-conjugated antibody. As shown in Fig. 4, incubation of AU-565 cells, naturally low in CAR (Fig. 5), with sCARfC6.5 protein increased cell binding of anti-CAR antibody. In contrast, neither the incubation of AU-565 cells with sCARf control protein nor the incubation of sCARfC6.5 with MDA-MB-468 c-*erb*B-2-negative cells revealed any increase of RmcB MAb antibody binding compared with MAb alone (Fig. 4). Thus, we demonstrated that C6.5 scFv that was incorporated in the context of fusion protein retained its functional ability to recognize cellular c-*erb*B-2 oncoprotein, which enabled sCARfC6.5 protein binding to c-erbB-2-positive cells.

sCARfC6.5 Adapter Protein Mediates c-*erb*B-2-specific Ad Infection. To test the utility of sCARfC6.5 adapter protein for Ad targeting, we evaluated its ability to improve Ad-mediated gene transfer to c-*erb*B-2-positive cells. The established human breast cancer cell lines AU-565, SK-BR-3, BT-474, MCF-7, and MDA-MB-468 and ovarian cancer cell line SK-OV-3 were used to validate the ability of c-*erb*B-2 oncoprotein to mediate Ad infection. Our previous study showed that these cells are relatively refractory to Ad infection.[4] The data were corroborated by flow cytometry analysis that showed either absence or low level of CAR on their cell surface (Fig. 5). Importantly, high levels of c-*erb*B-2 were detected in these cell lines (Fig. 5), which suggested that Ad targeting to c-*erb*B-2 may overcome poor vector susceptibility attributable to the lack of CAR. To determine the optimal adapter protein to virus ratio, sCARfC6.5 protein was titered against a constant dose of AdLucGFP vector (100 v.p./cell) as measured by improvements in gene transfer efficiency. The magnitude of gene transfer augmentation by targeted Ad complexed with sCARfC6.5 adapter was illustrated on selected c-*erb*B-2-positive cell lines and c-*erb*B-2-negative MDA-MB-468 cells compared with untargeted Ad preincubated with sCARf control protein or Ad alone (Fig. 6). Fig. 6*A* shows sCARfC6.5 protein dose-dependent enhancement of gene transfer that was achieved by targeted Ad compared with that achieved by untargeted Ad, as measured by luciferase activity that was detected in infected cells. The sCARfC6.5/Ad ratio providing maximal gene-transfer increase ranged from 1×10^{-7} to 3×10^{-7} pmol/v.p. depending on the cell line tested. As shown in Fig. 6*A*, the sCARfC6.5-targeting protein mediated a 3.4-, 11-, 32-, 47-, and 135-fold enhancement of gene transfer to MCF-7, SK-OV-3, BT-474, SK-BR-3, and AU-565 cells, respectively. The sCARfC6.5 adapter protein provided 1.5- to 17-fold increase of Ad gene transfer compared with Ad alone in most cell lines; however, the levels of improvement in cell transduction were highly variable (Fig. 6*B*). Although c-*erb*B-2-targeted Ad showed a 6-fold enhancement of gene transfer to SK-OV-3 cells and no transduction improvement of MCF-7 cells compared with Ad alone, both of the cell lines demonstrated similar levels of gene transfer and remained relatively Ad refractory. Consistent with the augmentation of the Ad gene transfer to c-*erb*B-2-positive cell lines achieved by sCARfC6.5 targeting protein, untargeted Ad that was complexed with sCARf control protein showed a marked decrease in gene transfer. Importantly, the use of both targeting and control protein to mediate the Ad infection of c-*erb*B-2-negative MDA-MB-468 cells that expressed moderate levels of CAR resulted in an 8-fold decrease of gene transfer. These data strongly indicate that the sCARfC6.5-targeting adapter promoted the Ad infection of CAR-deficient cells specifically via a c-*erb*B-2-dependent pathway.

Augmentation of c-*erb*B-2-targeted Ad Infection Efficiency. The Ad infection of the cells overexpressing c-*erb*B-2 oncoprotein mediated by the sCARfC6.5-targeting protein resulted in the enhancement of luciferase reporter gene expression. This increase in reporter activity could result from an increased number of infected cells or, alternatively, from an elevated level of transgene expression caused by the augmented infection of a limited population of cells. To address this issue, cells were infected with AdLucGFP vector, preincubated with PBS (Ad alone), sCARf (untargeted Ad), or sCARfC6.5 (c-*erb*B-2-targeted Ad) proteins at a sCAR protein:Ad ratio of 2×10^{-7} pmol/v.p. Ad infection efficiency was monitored by direct visualization of GFP expression by fluorescence microscopy. Fig. 7 shows the results of Ad-mediated GFP reporter gene delivery to three representative cell lines: AU-565, SK-BR-3, and BT-474. Infection with AdLucGFP vector alone resulted in a low percentage of GFP-expressing cells, whereas highly increased numbers of infected cells were detected in the case of c-*erb*B-2-targeted Ad. In contrast, infection

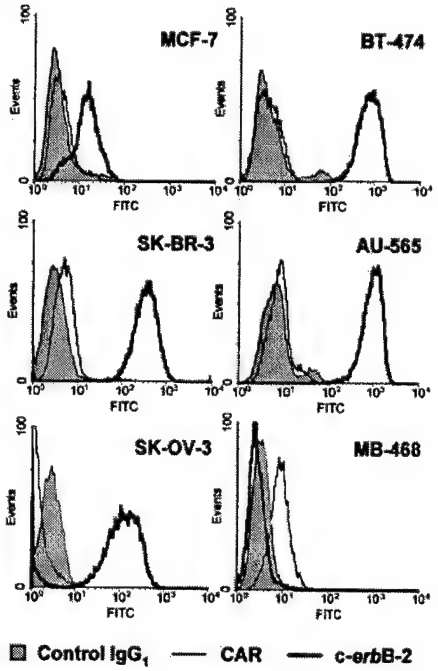

Fig. 5. Expression of CAR and c-*erb*B-2 in cancer cell lines. The breast cancer cell lines MCF-7, BT-474, SK-BR-3, AU-565, and MB-468 and ovarian cancer cell line SK-OV-3 were analyzed for CAR and c-*erb*B-2 expression (by indirect immunofluorescence assay using anti-CAR RmcB and anti-c-*erb*B-2/HER-2/neu Ab-2 MAb, respectively. Positive staining for CAR (*thin black line*) and c-*erb*B-2 (*bold black line*) is seen relative to an isotype control IgG (*spike filled in gray*). Representative data from two independent experiments are shown.

[4] Unpublished observations.

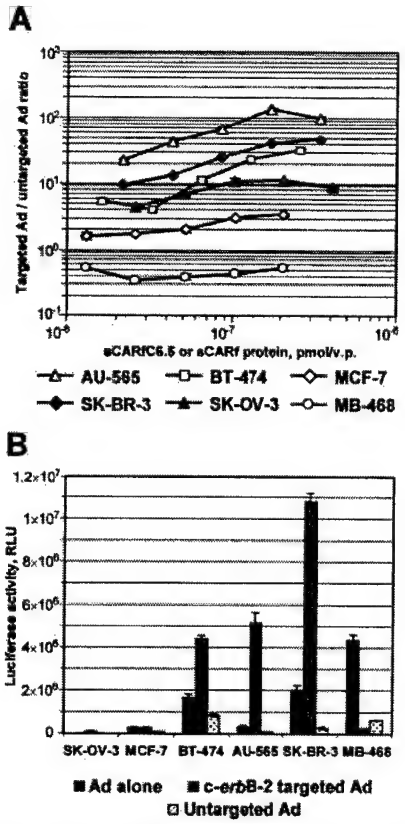

Fig. 6. sCARfC6.5 targeting adapter protein promotes Ad gene transfer to c-erbB-2-positive cells. *A*, determination of optimal sCARfC6.5 adapter protein:Ad ratio. AdLucGFP vector expressing luciferase reporter gene was preincubated with either sCARfC6.5 targeting protein or sCARf control protein at varying concentrations to form c-*erb*B-2-targeted or untargeted viral complexes, respectively. The monolayers of MCF-7, BT-474, SK-BR-3, AU-565, SK-OV-3, and MB-468 cells were infected with targeted or untargeted viral complexes at MOI of 100 v.p./cell. Cells were incubated for 46 h to allow expression of reporter gene, then were lysed, and the luciferase activity was analyzed. Results are presented as logarithm of ratio of luciferase activities detected in the cells infected with targeted Ad to luciferase activities detected in the cells infected with untargeted Ad complexes formed at the same concentration of each sCAR protein (*Targeted Ad:Untargeted Ad*). Each point represents the cumulative mean ± SD of triplicate determinations. *Error bars* (some are smaller than the symbols), SDs. *B*, enhancement of Ad gene transfer by sCARfC6.5 targeting protein. AdLucGFP vector was preincubated with either PBS (*Ad alone*) or one of sCARfC6.5 (*c-erbB-2-targeted Ad*) or sCARf (*untargeted Ad*) proteins at the concentration providing maximal gene transfer augmentation as determined in *A*. The cell monolayers were infected with Ad alone (■), c-*erbB*-2-targeted Ad (▣), or untargeted Ad (▨) viral complexes at MOI of 100 v.p./cell and analyzed for luciferase expression 46 h postinfection. Luciferase activities detected in cell lysates are shown as the cumulative mean of triplicate determinations of relative light units (*RLU*) ± SD.

with untargeted Ad showed a decreased infection efficiency compared with that of Ad alone, which resulted in few or no fluorescent cells. Infection of c-*erb*B-2-negative MDA-MB-468 cells with targeted Ad did not show any improvement of infection efficiency compared with that achieved by untargeted Ad or by Ad alone (data not shown). These results were consistent with data obtained for Ad-mediated luciferase gene delivery (Fig. 6).

DISCUSSION

One of the major challenges facing Ad gene delivery systems is the modification of viral native tropism to confer targeting capability on selected cell types. The limitation of Ad vectors associated with broad CAR-dependent tropism and inefficient infection of CAR-deficient cells could be solved by Ad targeting to a novel receptor overexpressed in the cells of interest. In this study, we explored the utility of a recombinant adapter protein to achieve modification of Ad tropism. The use of adapter molecules to mediate cell-specific Ad infection relies on the following: (*a*) the ability to be produced and purified at preparative amounts and retain stable structure; (*b*) the ability to provide efficient linkage to the v.p. while blocking Ad native tropism; and (*c*) the ability to achieve binding to cell-specific receptors, thus generating a novel tropism. We previously developed a targeting approach based on the use of the sCAR ectodomain fused with EGF, which allowed both the blocking of CAR-dependent tropism and the Ad targeting to the cells overexpressing EGFR (29). To extend this approach, we engineered an adapter protein, sCARfC6.5, comprised of sCAR fused with a trimerization domain and a scFv against c-*erb*B-2 oncoprotein to target cancer cell types, and a sCARf control protein that lacked the anti-c-*erb*B-2 scFv targeting moiety. A trimerization domain derived from phage T4 fibritin protein was incorporated into the design of the sCAR fusion proteins to achieve tight viral linkage by virtue of trivalent binding to the Ad fiber knob. Both the sCARfC6.5 targeting protein and the control sCARf protein were expressed in insect cells and purified at preparative amounts by affinity chromatography. An analysis of purified fusion proteins showed that sCARf and sCARfC6.5 retain their designed composition and maintain stable trimeric structure. As expected, trimerization of sCAR proteins resulted in the augmentation of knob-binding efficiency by at least 20-fold compared with that achieved by the monomeric sCAR-His_6 protein. This result is consistent with a kinetic analysis showing that, in contrast to the high on/off interaction rates between CAR D1 domain and Ad2 fiber knob, the binding of the knob domain to three D1 molecules simultaneously leads to a low overall off rate and K_d of ~1 nM (16). We then evaluated whether trimerization would result in an improved ability of sCAR proteins to block Ad infection. An infection inhibition assay demonstrated that the concentrations (3 and 6 nM, respectively) of trimeric sCARfC6.5 and sCARf proteins that were needed to inhibit Ad-mediated gene transfer by 50% were, respectively, 18- and 9-fold lower than the concentration (54 nM) of monomeric sCAR-His_6 protein that was needed. These data proved the utility of trimeric sCAR adapter proteins in blocking CAR-dependent Ad infection. The fact that T4 fibritin forms homotrimers that are resistant to dissociation by SDS and digestion by trypsin (37, 38) suggests that the incorporation of a fibritin polypeptide in the context of sCAR fusion might provide a highly stable trimeric structure compatible with *in vitro*, and likely *in vivo*, Ad targeting schemes. We showed that, in addition to its ability to block Ad infection, the sCARfC6.5 adapter protein binds to cellular c-*erb*B-2 oncoprotein and, therefore, enables Ad targeting via a CAR-independent pathway. In ovarian and breast cancer cell lines overexpressing c-*erb*B-2, the ability of the c-*erb*B-2 oncoprotein to mediate Ad infection was illustrated by markedly increased levels of gene transfer and numbers of infected cells. Thus, the use of the sCARfC6.5 adapter protein overcomes the barrier of CAR deficiency by retargeting the Ad infection via the c-*erb*B-2 oncoprotein and provides a ≤2-fold enhancement of gene transfer efficiency in comparison with that provided by the sCARf control protein. Our observation that the use of the sCARfC6.5 adapter decreased the Ad infection efficiency in c-*erb*B-2-negative MDA-MB-468 cells that expressed moderate levels of CAR suggests the high specificity of this Ad-targeting approach. Importantly, the presence of three scFvs in each trimeric sCARfC6.5 molecule likely contributes to the increase in apparent receptor affinity attributable to polyvalent binding and contributes, therefore, to the efficiency of Ad targeting. In fact, the presence of additional binding sites prolongs the association of scFv-based molecules with tumor cells *in vitro* and *in vivo*. Studies of bivalent diabody molecules that were constructed from C6.5 scFv demonstrated a more highly prolonged association (60-fold) with c-*erb*B-2

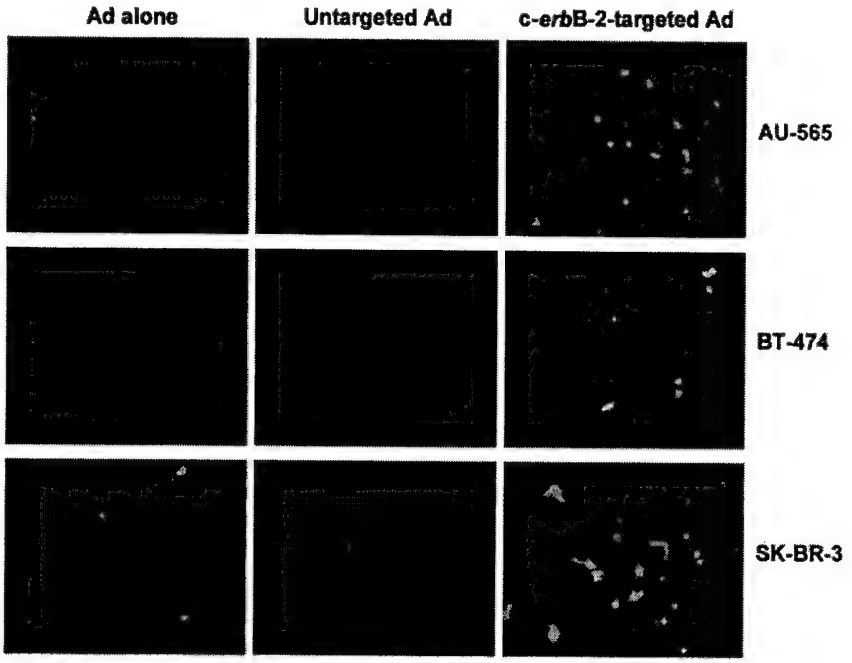

Fig. 7. The use of sCARfC6.5 targeting protein improves efficiency of Ad infection of c-erbB-2-positive cells. AdLucGFP vector expressing GFP was preincubated with PBS (*Ad alone*), sCARfC6.5 adapter protein, or sCARf control protein at the concentration of 2×10^{-7} pmol/v.p to form c-*erb*B-2-targeted or untargeted viral complexes, respectively. The monolayers of SK-BR-3, BT-474, and AU-565 cells were infected with Ad alone, untargeted Ad, or c-*erb*B-2-targeted Ad complexes at MOI of 100 v.p./cell. Infected cells expressing GFP were detected 24 h postinfection by fluorescence microscopy.

on the surface of SK-OV-3 cells and more tumor-retained diabodies (6.5-fold) when compared with scFv monomer (41).

The use of trimeric sCAR-ligand fusion proteins or recombinant adapter molecules sharing a trimeric design to confer Ad targeting to specific cell types may augment the utility of current Ad vectors. In addition, the availability of scFvs with defined specificities to tumor antigens offers the flexibility of ligand substitution to expand the targeting capabilities of Ad vectors. Of note, Ad targeting by means of bispecific antibody conjugates was shown to achieve direct therapeutic goals in *in vivo* models relevant to human clinical cancer gene therapy schemes (42–45). Thus, modification of Ad tropism based on the use of recombinant adapters could facilitate target-directed infection, which will reduce the effective therapeutic viral dose, thereby decreasing the immediate toxicity and increasing the safety and efficiency of Ad vectors.

ACKNOWLEDGMENTS

We are grateful to Dr. James D. Marks for the provision of the cDNA for C6.5 scFv, Dr. Robert Finberg (Dana Farber Cancer Institute, Boston, MA) for cDNA for CAR, and Dr. V. Mesyanzhinov for anti-fibritin serum. We thank Dr. Victor Krasnykh (Division of Human Gene Therapy, University of Alabama at Birmingham, Birmingham, AL) for the plasmid encoding recombinant Ad5 fiber knob protein and Dr. Joanne T. Douglas (Division of Human Gene Therapy, University of Alabama-Birmingham) for making RmcB antibody available to us. We thank the DNA Sequencing Core and the FACS Core Facility at the University of Alabama at Birmingham for providing assistance. Thanks to Dr. Joel N. Glasgow for fruitful discussions and proofreading of the manuscript.

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Gene Therapy (2002) 9, ●●●–●●●

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BRIEF COMMUNICATION

Coxsackievirus-adenovirus receptor genetically fused to anti-human CD40 scFv enhances adenoviral transduction of dendritic cells

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A promising approach to immunotherapy involves the loading of dendritic cells (DCs) with genetic material to facilitate sustained expression of a relevant antigen in this population of potent antigen presenting cells (APC). Viral vectors such as adenovirus (Ad) have been used for this purpose. Existing methods for DC infection are limited by lack of specificity and a requirement for DC exposure to high viral doses. Targeting of Ad to DCs with bispecific antibodies has significantly augmented levels of transgene expression. Genetic fusion of the extracellular portion of coxsackievirus-adenovirus receptor (CAR) to cell-specific ligands has also proved successful in targeting Ad to cells of interest. We report here the production and primary characterization of a new fusion protein comprising the ecto-domain of CAR connected to a single chain antibody (scFv) G28-5 against human CD40 present on the surface of DCs. We demonstrate that the fusion protein (CAR/G28) specifically interacts with both recombinant Ad fiber knob and the ecto-domain of human CD40 in a binding assay (ELISA). Finally, we show that the CAR/G28 fusion protein promotes highly efficient transduction of DCs of both rhesus monkey and human origin.

Gene Therapy (2002) **9**, ●●●–●●●. DOI: 10.1038/sj/gt/3301767

Keywords: *CAR; CD40; scFv; dendritic cells; adenovirus; transduction enhancement*

Dendritic cells play a central role in controlling immunity. Antigen is initially processed by immature DCs, which subsequently undergo maturation involving phenotypic and functional changes that enable DC to mediate specific T and B cell activation in a highly effective manner. In tumor-bearing hosts, antitumor immunity can be compromised because tumor antigens fail to elicit immune mechanisms to resist tumor growth. This outcome is possibly related to a deficiency of DC expressing tumor antigens. Accordingly, strategies to vaccinate against tumor-specific antigens utilizing DC are being developed to bolster anti-tumor immunity.[1]

Loading of DC with antigens can be achieved by incubation with antigen-specific peptides[2,3] or cell lysates.[3] Alternatively, antigen expression in DC can be induced by gene transfer using either RNA[4] or DNA with non-viral[1,5] or viral[1,6] vectors. Among the viral vectors, Ad is notable for efficient in vitro and *in vivo* gene transfer and expression, which is independent of target cell replication. However, the relative resistance of DCs to Ad infection due to lack of CAR expression on DC surface[7] is an ostensible shortcoming for this approach. Efficient DCs transduction with Ad has been reported, but high viral doses and prolonged exposure of the DCs to virus were required.[8] Ad transduction can also be facilitated with liposomes,[9] but toxicity for DCs is a potential problem. Thus, there is a need to improve the specificity of targeting Ad vectors to DC in the gene therapy field.

A recently reported approach to achieve directed Ad-based gene transfer to DCs utilized a chimeric Ad of serotype 5 in which the fiber knob region was replaced with that of Ad of serotype 35.[10] However, optimal transduction efficiency with such chimeric virus required a high virus-to-cell ratio (multiplicity of infection, MOI). An alternative approach of Ad targeting to DCs was developed in our laboratory using a chemical conjugate comprising a F(Ab) fragment of a monoclonal antibody raised against Ad serotype 5 fiber and a whole mAb against the DC marker protein CD40.[7] This bispecific antibody demonstrated high transduction efficiency of DCs at much lower viral doses. Notably, this DC-targeted, Ad infection strategy also lead to phenotypic changes reflecting DC maturation.

Recombinant proteins offer a number of technological advantages including simplified production and purification when compared with chemical conjugates. In addition, during chemical conjugation only a fraction of the input component will yield a functionally active

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Received 12 September 2001; accepted 12 March 2002

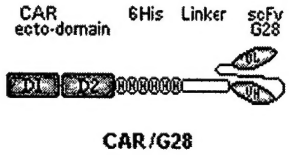

Figure 1 Structure of CAR/G28 fusion protein. The fusion protein consists of an extracellular portion of human CAR (domains D1 + D2) at the N-terminus connected to anti human CD40 scFv G28 via a short peptide linker (PSASASASAPGS) preceded by a 6 histidine (6His) purification tag. CAR binds to Ad5 fiber knob, whereas G28 binds to CD40 present on DCs, thus facilitating Ad viral docking to DCs.

bispecific molecule due to the inherent variability of the technique. A product intended for clinical use should have a strictly defined and reproducible composition. Recombinant proteins achieve this by rational design. We have recently described an approach of bispecific adaptor-based Ad targeting with a recombinant protein consisting of an extracellular portion of Ad receptor CAR fused to epidermal growth factor (EGF).[11] In that study, the CAR/EGF fusion protein specifically directed Ad infection to EGF receptor expressing cells that lacked expression of CAR. In the present study, our aim was to create a fusion protein between CAR and a single chain antibody against human CD40 (Figure 1). We hypothesized that such a recombinant CAR-anti-CD40scFv protein would facilitate efficient, specific DCs transduction with Ad.

The CAR/G28 fusion protein was generated by the following scheme. Initially, the extracellular domain of human CD40 (hCD40ecto, including amino acids 21–193, numbered according to Ref. 12) was produced in a prokaryotic expression system and purified. The anti-CD40 single chain Fv cDNA was generated from the G28-5 hybridoma cell line.[13] The hCD40ecto was then used to screen for CD40-specific scFv. The G28-5 scFv cDNA was linked to that of the CAR ecto-domain replacing the EGF portion in the CAR/EGF fusion protein.[11] Finally, the CAR/G28 fusion protein was produced using recombinant baculovirus, purified and characterized.

Recombinant hCD40ecto was produced in a prokaryotic expression system to facilitate anti-CD40 scFv and CAR/scFv isolation and validation. The hCD40ecto gene fragment was PCR-amplified from a cDNA clone generously provided by Dr I Stamenkovic (Molecular Pathol-

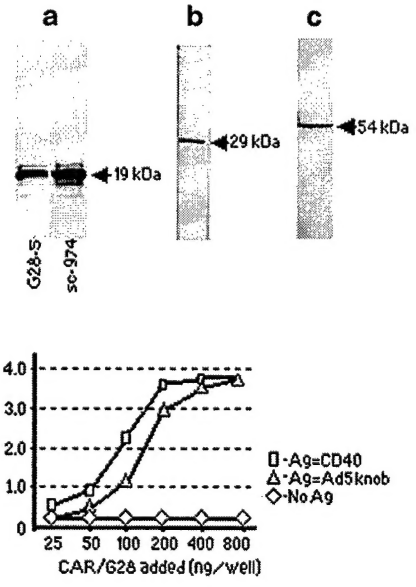

Figure 2 ●<aq1>● *Fusion proteins production and characterization. Production of ecto-domain of human CD40. To produce the hCD40ecto as a 6His-tagged recombinant protein, the corresponding gene fragment was PCR-amplified from a cDNA clone and ligated into pET21a expression vector (Novagen, Madison, WI, USA) using BamHI and NotI restriction sites. For the amplification, a pair of primers 5′ CTT GGA TCC GAA CCA CCC ACT GCA TGC AGA GAA A and 5′ ATT GCG GCC GCT CTC AGC CGA TCC TGG GGA CCA was used. The assembled plasmid was introduced into BL21(DE) E. coli cells and the gene expression was induced with IPTG. Produced protein was purified from cell lysate by immobilized metal affinity chromatography (IMAC) on Ni-NTA (Qiagen, Valencia, CA, USA). The purified protein (100 ng/lane) was separated on a 12% polyacrylamide-SDS gel (PAAG-SDS) followed by electrotransfer to a PVDF membrane. The membranes were probed with the hybridoma supernatant of mouse mAb G28-5 (ATCC No. HB9110) and with rabbit antibody sc-974 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), both specific for human CD40. (a) Construction of scFv G28-5 against human CD40. The G28-5 scFv was generated from a corresponding hybridoma using published sequences (Genebank accession numbers are AF013577 for VH and AF013576 for VL) for primer design. Hybridoma cells grown from a fresh clone were harvested and mRNA was isolated from 4×10^6 cells using an Oligotex Direct mRNA Micro Kit from Qiagen. Complimentary DNA was synthesized from this mRNA using an Omniscript reverse transcription kit from Qiagen and an Oligo(dT)15 primer from Promega (Madison, WI, USA). The VH and VL fragments were amplified from the cDNA and cloned into pSEX81 phagemid vector. Two pairs of primers G28HF: 5′ GAT ATA CAG CTT CAG CAG TC and Bi4: 5′ CCA GGG GCC AGT GGA TAG ACA AGC TTG GGT GTC GTT TT and G28LF: 5′ GAT GCT GTG ATG ACC CAA AAT and G28LR: 5′ GGA TAC AGT TGG TGC AGC ATC were used to amplify original VH and VL portions, respectively. The resulting phage display G28-5 mini-library was screened using the hCD40ecto recombinant protein. The CD40-positive phage clone 10 was selected from the mini-library, its gene re-cloned into pOPE101, introduced into XL1Blue E. coli cells (Stratagene), produced by IPTG induction and purified from periplasmic extract by IMAC on Ni-NTA. Panel (b) demonstrates an immunoblot of purified scFv G28 clone 10. The purified protein (20 ng/lane) was separated on 12% PAAG-SDS followed by electrotransfer to a PVDF membrane. The membrane was probed with mAb 9E10 (Sigma) against c-Myc epitope incorporated into the scFv. (c) Construction of CAR/G28 fusion protein. The scFv G28-5-10 cDNA fragment was amplified from pSEX81-G28-5-10 using a pair of primers 5′ CCG AGA TCTATA CAG CTT CAG CAG TCA GGA CCT and 5′ AGC GAG CTCCCG TTT TAT TTC CAG CGT GGT followed by digestion with BglII and SacI. The digested fragment was cloned in frame into the pFBsCARslEGF plasmid[11] replacing the EGF which was excised with BamHI and SacI. The new plasmid, pFBsCARslG28, encoding recombinant shCAR fused with scFv G28-5-10 (CAR/G28) and tagged with internal 6His was then used for generation of the recombinant baculovirus genome, according to recommendations for Bac-toBac baculovirus expression system (Life Technologies, Grand Island, NY, USA). All newly engineered cDNA constructs were examined by sequencing using CEQ2000 automatic sequencer and CEQ dye terminator sequencing kit from Beckman (Fullerton, CA, USA). (c) Immunoblot of purified CAR/G28. The purified protein was separated on 12% PAAG-SDS followed by electrotransfer to a PVDF membrane. The membrane has been probed with rabbit anti-CAR antibodies produced in the laboratory. Horseradish peroxidase (HRP)-labeled secondary antibodies and diaminobenzidine staining were used for binding visualization. (d) CAR/G28 fusion protein is bi-specific. CAR/G28 fusion protein binding to both hCD40ecto and Ad5 knob was examined in ELISA. The antigens were adsorbed on plastic (300 ng/well each) and probed with CAR/G28. Binding was detected with rabbit anti-CAR antibodies (1:3000, produced in-house) followed by HRP-labeled anti-rabbit antibodies and orthophenilenediamine staining. (d) CAR/G28 interaction with hCD40ecto and recombinant Ad5 knob.*

ogy Unit, Massachusetts General Hospital and Department of Pathology, Harvard Medical School, Boston, MA, USA). Next, the hCD40ecto cDNA was subcloned into the pET21a plasmid and expressed in BL21(DE) *E. coli*. The hCD40ecto recombinant protein of 19 kDa was purified from the cell lysate and its identity confirmed by immunobloting with polyclonal antibody sc-974 and mAb G28-5 against human CD40 (Figure 2a).

The gene segments coding for VH and VL of the G28-5 mAb were PCR-amplified from the corresponding hybridoma cDNA and ligated with a Yol linker by two-step cloning into pSEX81[14] phagemid, thus creating a miniphage display library that facilitated anti-CD40 scFv isolation. The library was screened using hCD40ecto as described.[15] Positive clones were identified in ELISA, and primary DNA structure of the scFv gene was confirmed by sequencing. Single chain Fv G28-5 cDNA was re-cloned into expression vector pOPE101[16] used to produce scFv G28-5 of approximately 29 kDa in *E. coli*. Purification of scFv was performed with Ni-NTA as described[17] (Figure 2b). The ability of scFv G28-5 to specifically bind hCD40ecto was demonstrated in ELISA (data not shown).

To generate the CAR/G28 fusion protein, scFv G28-5 cDNA was cloned in-frame with that of CAR, generating the pFBsCARslG28 plasmid, which was then used for baculovirus genome generation. Our attempts to express CAR/G28 cDNA in various bacterial expression systems failed due to massive protein degradation (data not shown). The baculovirus genome containing the CAR/G28 cDNA was used to transfect Sf-9 insect cells and the resultant baculovirus was used for larger scale infection of High Five cells. The recombinant fusion protein was produced and purified from 1 liter of supernatant of infected cell culture. Purified recombinant fusion protein had the expected molecular weight of 54 kDa as demonstrated by immunoblot with anti-CAR antibodies (Figure 2c). The level of the fusion protein production was approximately 2 mg/l of culture. Specificity of the CAR/G28 towards both recombinant hCD40ecto and Ad5 fiber[18] knob was demonstrated in ELISA (Figure 2d). After confirming the dual specificity of CAR/G28, we examined the recombinant protein for its ability to target Ad infection to DCs of rhesus monkey or human origin. Adenoviruses bearing luciferase (Luc) or green fluorescent protein (GFP) as transgenes were used in the DC transduction experiments.

Rhesus monocyte-derived DCs (RhMDDC) were derived from heparinized peripheral mononuclear cells isolated by gradient centrifugation. Monocytes were enriched to a purity of >80% by adherence to anti-CD14 coated magnetic microbeads and cultured in RPMI containing autologous plasma and cytokines recombinant human GM-CSF and IL-4 as described.[19] The phenotype of MDDCs was assessed by staining for HLA-DR, CD83, CD80 and CD86 with corresponding anti-human antibodies that cross-react with rhesus macaque (data not shown). The RhMDDCs were infected with Ad at MOI of 100 p.f.u./cell (optimal viral dose determined in Ref. 7) in the presence and absence of CAR/G28. Infection efficiency was assessed by measuring the transgene expression. As shown in Figure 3, both luciferase and GFP activity were significantly higher when the Ad infection was mediated by CAR/G28. The fusion protein enhanced DC transduction in a dose-dependent manner (Figure 3a, b). Of note, doses of CAR/G28 higher than 100 ng per 2.5×10^4 DCs caused further transgene expression enhancement, but some cell toxicity was

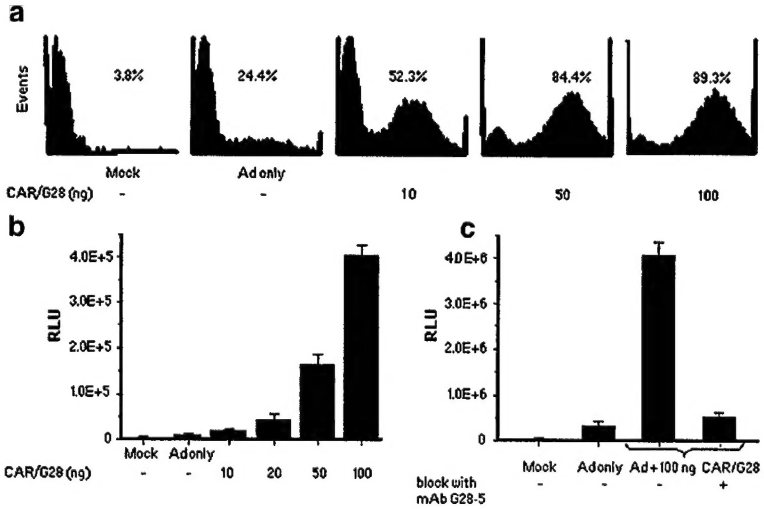

Figure 3 *Rhesus DCs transduction with Ad5. Rhesus MDDCs were obtained, briefly, as follows. Mononuclear cells were isolated from normal rhesus heparinized blood by Ficollpaque density gradient centrifugation. Monocytes were enriched by adherence to anti-CD14 magnetic microbeads (Miltenyi Biotec, Auburn, CA, USA). Monocyte-enriched cells were cultured at ~1 × 10^6 cells/ml in RPMI supplemented with 1% autologous plasma, 800 U/ml of granulocyte/macrophage colony-stimulating factor (GM-CSF; Immunex, Seattle, WA, USA) and 1000 U/ml of IL-4 to generate immature MDDCs. On day 6 immature RhMDDCs were infected with Ad5CMVluc (2.5 × 10^4 cells/experimental point) or Ad5CMVGFP (2 × 10^5 cells/experimental point) at MOI of 100 or 500, respectively. The virus was pre-incubated with indicated amounts of CAR/G28, before mixing with MDDCs. Transduced MDDCs were analyzed for GFP expression by flow cytometry to measure increased fluorescence intensity (a) or Luc content (b,c) by luminometry to measure relative light units (RLU). Specificity of transduction was demonstrated by inhibition of infection with G28-5 hybridoma supernatant. Mock, RhMDDCs were treated with neither virus, nor fusion protein; Ad only, RhMDDCs were treated with Ad without the fusion protein.*

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observed at those doses (data not shown). This toxicity effect is probably associated with an unknown effect of soluble CAR. Pre-treatment of DCs with G28-5 hybridoma supernatant efficiently blocked the infection (Figure 3c), indicating that Ad infectivity enhancement was due to engagement of CD40 for virus docking. These results suggest that the dual (human and rhesus) reactivity of CAR/G28 provides an advantage for DC-based vaccine approaches using gene therapy in the nonhuman primate model.

We also evaluated Ad transduction of human DCs with CAR/G28 fusion protein. Human monocyte-derived (HMDDC) were obtained by gradient centrifugation followed by plastic adherence enrichment and cultivation in the presence of cytokines.[20] The HMDDCs were infected with Ad in the presence and absence of indicated amounts of CAR/G28. The ability of CAR/G28 to enhance Ad infectivity towards human DCs was examined with adenovirus vectors bearing either Luc or GFP reporter genes. GFP expression was assessed by direct fluorescence microscopy, whereas the Luc expression was indirectly measured on a luminometer. Data presented in Figure 4 demonstrate CAR/G28-mediated enhancement of Ad transgene expression assayed both qualitatively (Figure 4a–c) and quantitatively (Figure 4e). As a positive control, the Fab-α-CD40 chemical conjugate[7] was used (Figure 4d, f). Analysis of data presented in Figure 4 suggests that the CAR/G28 fusion protein achieves similar levels in promoting DC transduction compared with the Fab-α-CD40 chemical conjugate.

In summary, we describe here a novel recombinant fusion protein designed to promote specific adenovirus-mediated transduction of DCs. After examining prokaryotic and eukaryotic expression systems, we achieved acceptable levels of CAR/G28 recombinant fusion protein production using baculovirus-infected insect cells. The fusion protein has a polyhistidine tag, allowing efficient purification. From the production standpoint, this feature makes the recombinant fusion protein superior in comparison to the anti-Ad:anti-CD40 bispecific antibody Fab-α-CD40 prepared by chemical conjugation. We have demonstrated recently the feasibility of a CAR-ligand fusion protein as an efficient Ad targeting moiety.[11] The present work expands the utility of this kind of CAR-containing molecular adaptor to a new level with scFvs as a fusion partner. Current gene engineering technology allows generation of scFvs against virtually any antigen. The CAR/G28 fusion protein demonstrated the expected dual binding specificity and substantially enhanced Ad gene transfer to DCs (up to 200 times in some experiments compared with untargeted Ad). Monoclonal antibody G28-5 has been shown to be internalized upon CD40 binding.[21] Therefore, we have reason to believe that this internalization process can be involved in CD40-targeted Ad infection of DCs mediated by CAR/G28, in addition to normal, integrin-dependent, viral entry to cells. From a functional perspective, the fusion protein has been tested in two distinct experimental systems using rhesus monkey and human DCs. The cross-reactivity of the CAR/G28 fusion protein with CD40-positive human and monkey DCs resulted in marked enhancement of Ad infection in both systems. These data establish the feasibility of using the nonhuman primate rhesus macaque model for preclinical studies with CAR/G28 to advance gene therapy-based DC vaccine development. In both systems the fusion protein demonstrated outstanding ability to target Ad vectors to DCs efficiently and specifically. The development of

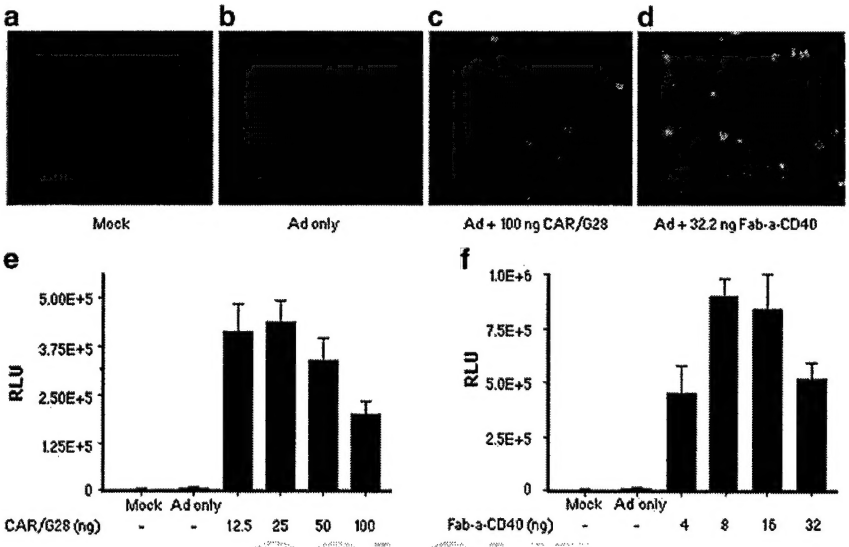

Figure 4 Human DCs transduction with Ad5. Human DCs were generated from peripheral blood monocytes obtained from healthy donors. Monocytes, purified by adherence, were cultured for 6 days in Iscoves's medium (Mediatech Cellgro, Herdon, VA, USA) with 10% human AB serum (Sigma, St Louis, MO, USA) containing 200 ng/ml of interleukin-4 (IL-4; R&D Systems, Minneapolis, MN, USA) and 1000 U/ml of GM-CSF. The resulting DCs were washed in phosphate buffered saline and resuspended at 5 × 10^5/ml in a serum-free medium, macrophage SFM (Gibco BRL, Grand Island, NY, USA) for infection. Human MDDCs were transduced essentially as described in the legend to Figure 3, with the exception that Ad5CMVGFP was used at MOI of 100 and the cells were assayed by fluorescent microscopy. CAR/G28 comparison with Fab-α-CD40 has been done for both viruses. (a) Both CAR/G28 and Fab-α-CD40 potentiate MDDCs transduction with Ad5CMVGFP. (b,c) Both CAR/G28 and Fab-α-CD40 potentiate MDDCs transduction with Ad5CMVluc.

CAR/G28 presents an opportunity to improve existing Ad-based approaches to genetically modify DCs for the treatment of human diseases.

Acknowledgements

This work was sponsored by the grant DAMD17-00-1-0115 from United States Army Department of Defense and the grants R01 CA86881, R21 AI44322, U19 DK57858, N01 CO-97110 from the National Institute of Health. The authors would like to thank Drs Paul Reynolds and Laura Timares for fruitful discussions.

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